University of Torino

Department of Medical Sciences

PhD in Biomedical Sciences and Oncology

Curriculum: Advanced Immunodiagnostics

Cycle XXXIII

BIOLOGICAL ROLE OF CD157 IN THE MODULATION OF ACUTE MYELOID LEUKEMIA CELL SURVIVAL AND CHEMOTHERAPY SENSITIVITY

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Academic Years 2017-2021

SSD: MED/03

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Abstract

Acute myeloid leukemia (AML) is a clinically and genetically heterogeneous disease characterized by block of myeloid differentiation, and clonal expansion of immature myeloid blasts within the bone marrow (BM) and peripheral blood. It is one of the most aggressive forms of leukemia being prevalent among elderly individuals. Despite advances in the treatment of AML, prognosis of some AML patients is still dismal due to development of chemoresistance and tumor relapse frequently overlapping with deregulation of the apoptotic machinery.

CD157, an adhesion molecule expressed by myelomonocytic cells, bone marrow stromal cells (BMSCs) and selected epithelial cancers, was found expressed in more than 90% of AML patients both at diagnosis and relapse, especially in M4 and M5 subtypes. In this study we used peripheral blood and BM samples from AML patients at diagnosis in order to analyse the impact of CD157-directed antibodies in AML survival and in response to cytarabine (AraC) ex vivo. The study was extended to the U937, THP1 and OCI-AML3 AML cell lines of which we engineered CD157low versions by shRNA knockdown. CD157-targeting antibodies elicited a time- and dose-dependent pro-survival effect, decreased apoptosis and reduced AraC toxicity in AML blasts and cell lines. CD157-mediated signaling activated the PI3K/Akt/mTOR and MAPK/ERK pathways, and increased expression of the Mcl-1 and Bcl-XL anti-apoptotic proteins, while decreasing expression of Bax pro-apoptotic protein, thus preventing Caspase-3 activation. The primary CD157-mediated anti-apoptotic mechanism was Bak sequestration by Mcl-1. Indeed, S63845 (a specific Mcl-1 inhibitor) restored apoptosis by disrupting the interaction of Mcl-1 with Bim and Bak, and significantly increased AraC toxicity in CD157high but not in CD157low AML cells.

Genetic loss of CD157 in AML cell lines reduced cell adhesion to selected extracellular matrix proteins, and attenuated the protective effect exerted both by fibronectin and BMSCs against AraC-induced cell death. CD157 expressed by HS-5 BM stromal cells participates to AML maintenance and protection driving AML cells to accumulate in G0/G1 cell cycle phase, thus contributing to cell-adhesion mediated drug resistance.

Collectively, these data demonstrated a new role of CD157 in AML cell survival and drug sensitivity, and indicated that CD157 is a promising candidate as predictive marker of response to therapies exploiting Mcl-1 pharmacological inhibitors. Moreover, by virtue of its role in the interactions between leukemia and the microenvironment,

CD157 could offer the opportunity to simultaneously target tumor cells and their environment.

1. Introduction

1.1 Acute myeloid leukemia

Acute myeloid leukemia (AML) is the most common acute leukemia in adults, and is associated with the highest number of death among leukemias. In 2021 alone, it is estimated that there will be 20,240 new cases of AML, and 11,400 people will die of this disease in United States [1]. Although AML can occur in any age, it is predominantly an older adults disease, with a median age at diagnosis of 68 years [2], and its incidence increases with age, with a slight prevalence in male, compared to woman [3].

AML is a genetically, epigenetically, and clinically heterogeneous disease characterized by accumulation and expansion of immature myeloid cells in the bone marrow (BM) and peripheral blood (PB), with consequent impaired hematopoiesis and aggressive clinical course [4]. Advances in genomic and in stem cell biology characterization of AML have fostered better understanding AML pathogenesis mechanisms. Although it is still not completely understood, AML is believed to originate from the oncogenic transformation of a haemopoietic stem cell or of progenitors that have reacquired stem cell-like properties of self-renewal [5]. These rare leukemic stem cells are quiescent, making them particularly resistant to cytotoxic chemotherapy and contributing to relapse [6].

Although the prognosis of AML patients has improved over the last decades with median overall survival (OS) at 5 years for patients younger than 60 years of approximately 40% [7], the long-term outcomes in older adults (aged >60 years) with AML are dismal with 5 year survival rates of around 15% [8]. Despite an increase in understanding the biology of AML, during the last decade only a mild improvement has been registered in term of OS and treatment options [9]. In this light, the identification of new targets for the therapy of this disease is considered a high priority.

1.1.1 Diagnosis and morphology

Morphologically, blasts are larger than lymphocytes and can be similar to monocytes or can be even larger [10]. A diagnosis of AML consists of morphological assessment of bone marrow or peripheral blood of 20% or more myeloid blasts (for

example, myeloblasts, monoblasts, or megakaryoblasts) [2, 11]. In addition, immunophenotyping by flow cytometry is used to further characterize the origin of malignant blast, and to confirm the AML subtype. AML blasts express antigens found also on healthy immature myeloid precursors, including common differentiation (CD) markers CD13, CD33, CD34, CD117, and HLA-DR [11, 12]. Depending on the stage of differentiation block and morphological subtype, blasts express monocytic differentiation markers (CD14, CD11b, CD36), granulocytic markers (CD15, CD65), or megakaryocytic markers (CD41a, CD61). CD38 and other markers such as CD123 or CD133 can be added to identify leukemic stem cells, but do not contribute to diagnosis [10-12]. Cytogenetics and screening for most common gene mutations is mandatory in the evaluation of suspected AML, and together with bone marrow aspirate morphology assessments and immune-phenotyping, remain essential for diagnosis, classification and risk stratification of AML patients [13].

1.1.2 Classification

Over the past years, there has been numerous attempts to classify AML based on morphology, phenotype and genetics. Two of the main systems has been used to classify AML into subtypes: French-American-British (FAB) classification and more recent World Health Organization (WHO) classification.

In the 1976, a group of French, American, and British experts divided AML into subtypes, from M0 to M7, based on the type of cell from which leukemia develops and its level of maturation (Table 1) [14]. The FAB classification can be useful, but it does not consider many factors which are now known to affect AML prognosis, such as genetic abnormalities and molecular features.

FAB subtype	Name
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyeloblastic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4 eos	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythdoid leukemia
M7	Acute megakaryoblastic leukemia

Table 1. French-American-British (FAB) classification of AML subtypes. Modified from the American Cancer Society cancer.org/1.800.227.2345

The WHO classification of AML replaced the old FAB classification and has been updated in 2017 (Table 2) [11]. It identifies AML subgroups defined by the presence of specific recurrent genetic abnormalities, including single mutations, gene fusions, or translocations [15].

Acute myeloid leukemia (AML) and related neoplasms
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22.1); RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
Acute promyelocytic leukemia with PML-RARA
AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A
AML with t(6;9)(p23;q34.1); DEK-NUP214
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1)
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1
Provisional entity: AML with BCR-ABL1
AML with mutated NPM1
AML with biallelic mutations of CEBPA
Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
AML, not otherwise specified (NOS)
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

Table 2. World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia. Modified from Dhner H. et.al, Blood 2017.

1.1.3 Cytogenetics and molecular abnormalities

Specific mutations occur at the early stage of leukaemogenesis and might provide a selective advantage for clonal expansion and eventual progression of the disease [16]. In details, some epigenetic mutations, like DNMT3A, TET2, and ASXL1 have been identified as early events that precede the development of AML [2, 17]. Around 10% of patients with AML develop the disease after the prior exposure to cytotoxic chemotherapy, or ionizing radiation, as consequence of treatment for primary malignancy [18, 19]. However, in most cases, AML appears as a *de novo* malignancy [17].

Great efforts have been made in understanding genetic heterogeneity of AML and how these aberrations affect disease biology, phenotype, response to therapy, and risk to relapse [20]. Recently, the advent of next generation sequencing (NGS) has allowed for genomic profiling leading to the identification of the AML mutational landscape [21]. In The Cancer Genome Atlas (TCGA) project 23 genetic mutations were found. Subsequently these mutations were classified in into 9 functional genetic classes involved in the pathogenesis of myeloid neoplasms [22]. Recently, NGS analysis of a cohort of 1540 AML patients described 11 mutually exclusive subgroups of genetic alterations with different clinical outcomes [23]. In addition to common AML mutations, such as FLT3 (internal tandem duplications, ITD, and tyrosine kinase domain mutations, TDK), K/NRAS, TP53 and c-Kit, or the presence of an NPM1 or biallelic CEBPA mutations, three additional categories were added. These included AML with mutations in genes encoding chromatin and RNA-splicing regulators, AML with TP53 mutations and/or cytogenetically visible copy number alterations, and AML with IDH2 R172 mutations [21, 23]. Together, genomic analysis has uncovered the alterations and clonal evolution of AML genetic profile during disease progression. Nevertheless, we are far from knowing AML genetics in detail, as leukemias are highly heterogeneous not just between patients but also within patients [16].

1.1.4 Risk stratification

After the diagnosis of AML has been made, the prediction of patient's outcome is made, based on the presence of cytogenetic and molecular aberrations predicting response to chemotherapy and risk to relapse [24]. In 2017, the European LeukemiaNet (ELN) has updated the risk stratification of AML patients by incorporating chromosomal and genetic

abnormalities. Patients are classified into favorable, intermediate, or adverse risk group (Table 3). For example, favorable prognosis is associated with mutated NPM1 without FLT3-ITD, or biallelic CEBPA. Intermediate category includes cytogenetic abnormalities neither favorable nor adverse, while complex karyotype is associated with poor prognosis [11]. Studies have shown that age and poor performance status is an independent predictor of poor outcomes regardless ELN classification [25]. The picture is more complicated when analyzing different cytogenetic and molecular landscapes in patients with relapsed and/or refractory (R/R) AML with OS estimated with less than 10% at 3 years [26].

Risk category	**Genetic abnormalities**
Favorable	t(8;21)(q22;q22.1); RUNX1-RUNX1T1 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11 Mutated NPM1 without FLT3-ITD or with FLT3-ITDlow Biallelic mutated CEBPA
Intermediate	Mutated NPM1 and FLT3-ITDhigh Wild-type NPM1 without FLT3-ITD or with FLT3-ITDlow (without adverse-risk genetic lesions) t(9;11)(p21.3;q23.3); MLLT3-KMT2A Cytogenetic abnormalities not classified as favorable or adverse
Adverse	t(6;9)(p23;q34.1); DEK-NUP214 t(v;11q23.3); KMT2A rearranged t(9;22)(q34.1;q11.2); BCR-ABL1 inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2,MECOM(EVI1) 25 or del(5q); 27; 217/abn(17p) Complex karyotype, monosomal karyotype Wild-type NPM1 and FLT3-ITDhigh Mutated RUNX1 Mutated ASXL1 Mutated TP53

Table 3. 2017 European LeukemiaNet (ELN) risk classification of AML. Modified from Dhner H. et.al, Blood 2017

1.1.5 Therapy

Although the molecular heterogeneity of AML has become better understood over the past decade, for more than 50 years the standard treatment of AML has consisted of a combination of cytarabine (AraC) and anthracycline, in a standard intensive regimen "7+3" chemotherapy [27], followed by post-remission consolidation therapy and allogenic hematopoietic stem cell transplantation (SCT), in eligible patients [7]. This regimen may induce a complete remission (CR) in 60-80% of younger adults, and 40-60% among older adults over 60 years old [28]. Most patients with AML who achieve a CR will eventually relapse due to the existence of subpopulations of AML cells resistant to standard chemotherapy [29]. Attempts to overcome or prevent resistance have led to the development of drugs targeting specific mechanisms of resistance to be administrated with traditional intensive and lower intensity chemotherapy regimen or as single or combination targeted therapies [24, 27, 28, 30].

With the advent of NGS that allowed more comprehensive profiling of the AML mutational landscape, the last few years saw landmark approvals of multiple targeted therapies for AML patients, including midostaurin in combination with 7+3 standard induction chemotherapy for the treatment of adults with newly diagnosed FLT3-mutated AML [31]; the re-approval of gemtuzumab ozogamicin, the antibody-drug conjugate combining monoclonal antibody (mAb) against CD33 and calicheamicin, a DNA damaging toxin, for newly diagnosed CD33-positive AML patients in combination with the intensive chemotherapy [32]; and small molecule inhibitors for both mutant IDH1 (ivosidenib) and IDH2 (enasidenib) approved for patients with R/R IDH1 or IDH2 mutated AML, respectively [33, 34].

As the majority of AML patients are ≥68 years at diagnosis, older patients represent the large proportion of AML patients. Studies show that a substantial proportion of older patients with AML do not receive any antileukemia therapy, as 7+3 intensive therapy is associated with poor outcomes, both in terms of toxicities and low responses and survival [8]. Consequently, for older patients less intensive therapies (e.g., low dose cytarabine or hypomethylating agents) are frequently preferred, although they provide only modest levels of benefit [27, 29].

1.1.6 BH3 mimetics in AML therapy

Aberrant overexpression of members of the BCL-2 family proteins is associated with tumorigenesis and increased resistance to chemotherapy in multiple malignances, including AML [35]. The pro-survival BCL-2 family proteins, such as BCL-2, BCL-XL, and Mcl-1 are proposed to function by binding and sequestering the pro-apoptotic BH3-only proteins, such as Bim, which in turn prevent the effector proteins, Bax and Bak, activation, ultimately preventing the permeabilization of the mitochondrial outer membrane and the subsequent induction of caspase-mediated apoptosis [36, 37]. As the major regulators of the intrinsic mitochondrial pathway of apoptosis, the BCL-2 family members are attractive targets for novel cancer therapeutics. Recently, the treatment landscape for AML has expanded significantly, thanks to the development of the small molecule inhibitors known as BH3 mimetics, targeting selected members of the anti-apoptotic BCL-2 family. By virtue of their structural similarity to the BH3 domains of the BH3-only proteins, these drugs displace the latter, and induce apoptosis trough Bax/Bak activation [38]. While many compounds have initially failed to specifically inhibit BCL-2 related proteins, ABT-199 (Venetoclax), a selective inhibitor of BCL-2, has demonstrated encouraging results in combination therapy, receiving ultimately a full FDA approval in 2020 for the use in combination with low-dose cytarabine or hypomethylating agents for patients with newly diagnosed AML in patients ≥75 years-old or younger who are ineligible for intensive chemotherapy [39].

Although the addition of venetoclax to AML therapies induced improved response rates and survival for many patients, most patients who do not reach allogenic SCT will eventually become resistant to therapy. One of the main resistance mechanisms to BCL-2 inhibition in AML is reliance on alternative antiapoptotic proteins, such as Mcl-1 and BCL-XL [39]. In particular, Mcl-1 overespression has been shown to be an extremely challenging mechanism of resistance to BCL-2 inhibition [40]. Unlike other BCL-2 family members which are long-lived proteins, Mcl-1 has a short half-life, and is highly regulated at transcriptional and post-transcriptional levels in response to various stimuli [41]. Initially, directly inhibiting Mcl-1 with BH3 mimetic compounds has proven difficult due to its shallow BH3-domain binding pocket. Early efforts to identify selective Mcl-1 inhibitors, led to the development of the small molecule inhibitor, S63845 [42]. S63845 is a highly selective Mcl-1 antagonist able to disrupt the Bim/Mcl-1 interaction that displayed high on-target activity in preclinical models, and induced Bax/Bak-

dependent apoptosis [42, 43]. Currently, early clinical trials with specific Mcl-1 inhibitors (S64315, AMG176, and AZD5991) have been launched (NCT02675452, NCT02979366, NCT03218683), aimed to assess these specific inhibitors as single agents, or though combination therapy with BCL-2 inhibitors [43].

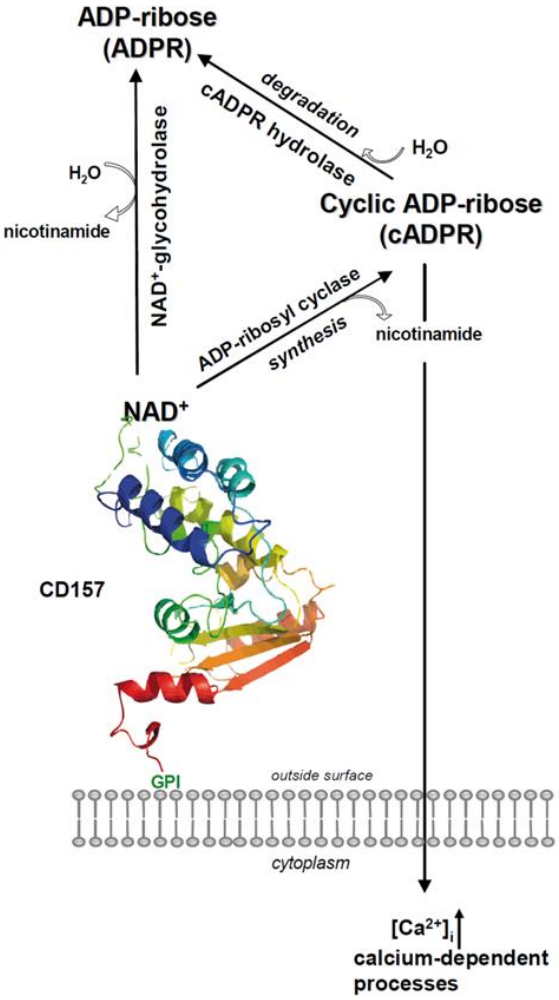
The importance of BCL-XL as target in AML has been less investigated. Similar to Mcl-1, BCL-XL represents a potential escape mechanism for survival following BCL-2 inhibition. However, clinical use of the dual BCL-2/BCL-XL inhibitor ATB-236 (navitoclax) was limited due to occurrence of thrombocytopenia, as platlets are particularly dependent on BCL-XL for survival [39]. In order to reduce the toxicity of navitoclax, new technology has been employed, converting navitoclax into DT2216, a Bcl-XL proteolysis-targeting chimera (PROTAC) that targets Bcl-XL to the Von Hippel-Lindau (VHL) E3 ligase for degradation, which is only poorly expressed in platelets, while its therapeutic potential remains similar to that of the original drug [44].

1.2 The CD157 protein

CD157 is a Glycosylphosphatidylinositol (GPI)-anchored glycoprotein first described over three decades ago as Mo5, a human myelomonocytic differentiation antigen [45], and later identified on the surface of human Bone Marrow Stromal Cells (BMSCs) and subsequently named Bone Marrow Stromal antigen 1 (BST-1) [46]. In the VI Workshop on Leukocyte Differentiation Antigens, Mo5 and BST-1 were grouped together as CD157 [47]. CD157 belongs to the NAD glycohydrolase (NADase)/ADP ribosyl cyclase ADPRC mammalian gene family, together with its paralogous gene CD38, with which it clusters in a head-to-tail manner on chromosome 4. CD38 and CD157 show a significant homology in their amminoacid sequence, but differ in their protein structure, as CD38 is a type II transmembrane glycoprotein with a small intracytoplasmatic region, while CD157 is a type I glycoprotein anchored to the cell membrane by the GPI moiety (Figure 1) [48]. Moreover, CD157 is much less efficient ADP-ribosyl cyclase than CD38, and its enzymatic functions are pH-dependent and require metal ions [49].

CD157 protein molecular weight ranges between 42-50 kDa, due to its heterogeneous glycosylation pattern [50]. In addition to the GPI-anchored form, CD157 exists in soluble form, measurable in serum and other biological fluids [51, 52]. Besides

soluble protein generated by proteolytic cleavage of the membrane-bound form, CD157 has also been found on exosomes released by myeloid-derived suppressor cells [53], and by mesothelioma cells [52]. However, the functional role of soluble CD157 remains to be defined.



Funaro A. et al, Frontiers in Bioscience, 2009

Figure 1. Schematic representation of CD157 protein structure and catalytic reactions. CD157 is a GPI-anchored protein. CD157 has both ADP-ribosyl cyclase and cADPR hydrolase activities: it catalyzes the production of cyclic ADP-ribose (cADPR) and ADP-ribose (ADPR), starting from its substrate NAD^+.

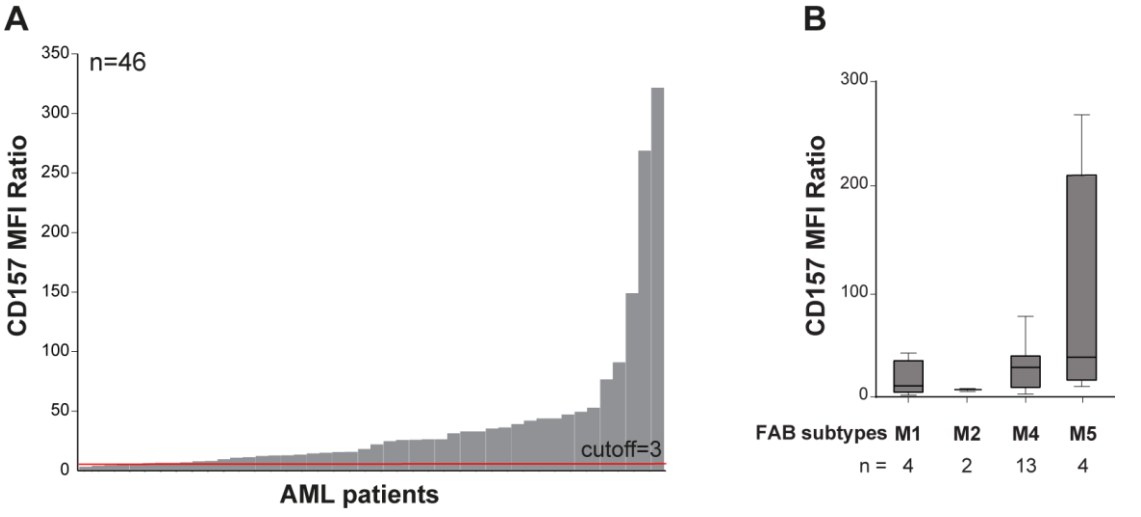
1.2.1 CD157 expression in health and disease

Human CD157 protein is prevalently expressed by cells of the myelomonocytic lineage, especially in monocytes, neutrophils and its expression increases with myeloid cell differentiation, hence its description as myeloid differentiation antigen [45, 48]. However, CD157 has actually a broader expression pattern as previously thought. Indeed,

it is expressed by several other cell types of both lymphoid and non-lymphoid origin, including myeloid-derived suppressor cells, mesenchymal stromal cells, endothelial cells, dendritic follicular cells and Paneth cells [54]. CD157 has been reported by our group to be expressed in epithelial ovarian cancer and malignant pleural mesothelioma, where high CD157 levels were correlated with tumor aggressiveness *in vitro* and *in vivo* [55, 56].

In hematological malignances, CD157 has been proposed as prognostic marker in B-cell precursor acute lymphoblastic leukemia useful for disease monitoring [57]. In acute myeloid leukemia, CD157 is expressed at variable levels by 97% of AML patients (Figure 2A), with no variation of expression at diagnosis or relapse [58]. The highest expression of CD157 is associated with M4 and M5 FAB classification subgroups (myelomonocytic and myelocytic leukemia) respect to M1 and M2 (Figure 2B) [58, 59]. A correlation between high CD157 surface expression and the adverse prognosis group of patients (according to the ELN classification 2017 [11]), has been reported. While, no correlation was found between NPM1 and FLT3-ITD mutational status and CD157 expression in a small AML cohort analysed [58].

Though at lower extent than in bulk AML blasts, CD157 was also found to be expressed in $CD34^{+}CD38^{-}$ leukemia-initiating cells, characterized by long-term repopulating potential, ability to propagate and maintain the AML phenotype, and are believed to be critical for AML relapse [58, 60].

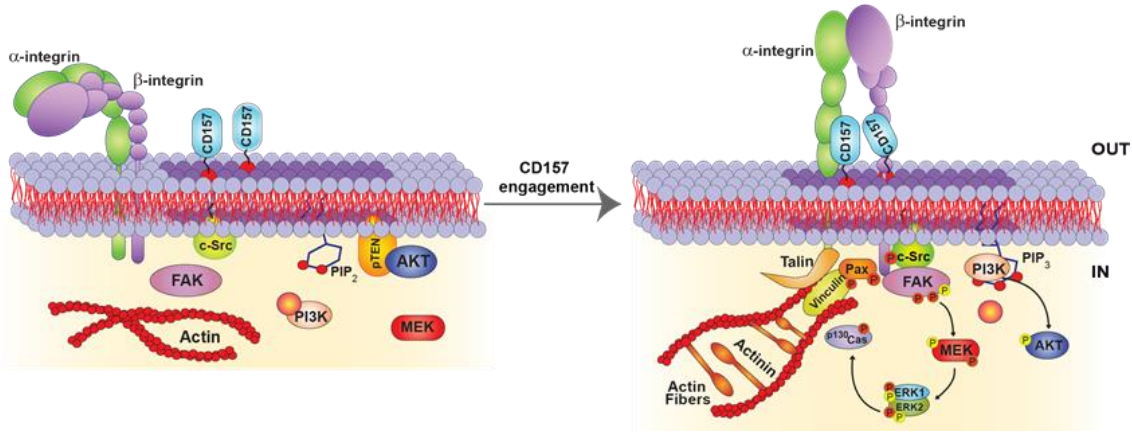


Yakymiv Y. et al, Cells, 2019

Figure 2. CD157 expression in acute myeloid leukemia. A) Flow cytometry analysis of CD157 expression of 46 AML patients at diagnosis. **B)** CD157 Mean Fluorescence Intensity (MFI) ratio correlated to FAB subtypes in 23 bone marrow samples from AML patients. MFI values of CD157 were normalized to the MFI of lymphocytes (which are CD157-negative), within each sample. Samples with CD157 MFI ratio ≥ 3.0 were considered as positive.

1.2.2 CD157 protein receptorial activity

Besides its functions as an ectoenzyme, CD157 is able of transducing intracellular signals although it lacks a cytoplasmic domain. To overcome its structural limitations, CD157 exploits the lateral mobility provided by the GPI-anchor to establish functional and structural interactions with β1 (CD29) and β2 (CD18) integrins [61]. Upon antibody-induced clustering (mimicking its engagement by a non-substrate ligand), CD157 promotes the recruitment of β1 and β2 integrin into lipid rafts, thus inducing the formation of a multimolecular complex favoring signal transduction. Indeed, CD157-mediated intracellular signaling relies on the integrin/FAK/Src pathway, leading to increased activitation of downstream MAPK/ERK1/2 and PI3K/Akt signaling pathways, which are involved in the control of monocyte transendothelial migration (Figure 3) [62].



Modified from Lo Buono N. et al, Frontiers in Bioscience, 2013

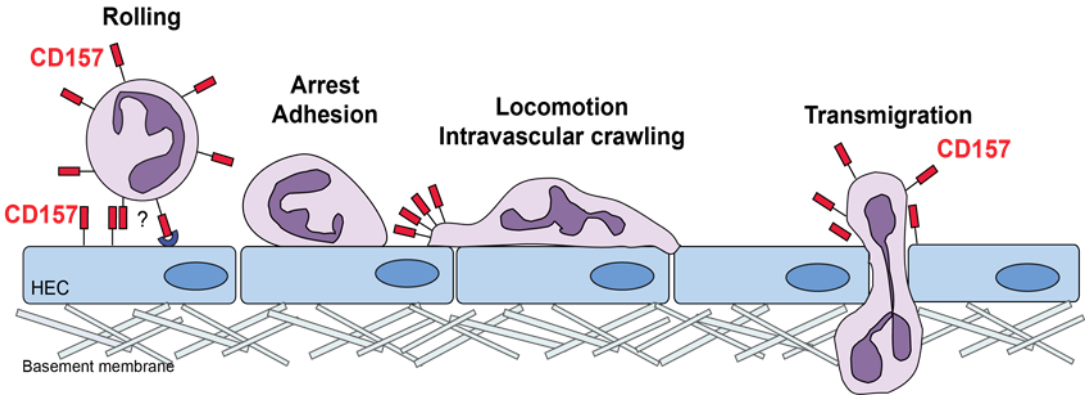
Figure 3. CD157 and integrin partnership. CD157 engagement by its non-substrate ligand recruits integrins into signaling-competent microdomains (lipid rafts), thus influencing their three-dimensional organization and promoting the assembly of a network of interconnected signal transduction pathways. CD157 and β1 and β2 integrins converge on activation of Src family kinases, which leads to increased activity of downstream MAPK-ERK1/2 and PI3K-Akt pathways, known to regulate cell adhesion and migration.

Moreover, CD157 engagement regulates calcium homeostasis and promotes polarization in neutrophils, and mediates superoxide (O2-) production in the human myelo-monocytic cell line U937 [47, 63]. However, the relationship between the enzymatic activities and receptorial functions of CD157 are still unknown and deserve further investigation.

1.2.3 CD157 role in the immune response

The constitutive expression of CD157 in neutrophils, monocytes and vascular endothelial cells (mainly at the interendothelial junctions) [64], was highly suggestive of its possible involvement in leukocyte migration.

Experimental evidence indicated that CD157 is a crucial player in the control of leukocyte trafficking during inflammation. Our group has demonstrated that CD157 regulates: i) neutrophil and monocyte adhesion to extracellular matrix proteins, ii) directional migration of both neutrophils and monocytes induced by chemoattractant factors and iii) neutrophil and monocyte transendothelial migration by establishing a structural and functional cross-talk with β1 and β2 integrins (Figure 4) [62, 63].



Modified from Funaro A. et al, Frontiers in Bioscience, 2009

Figure 4. CD157 controls key steps of leukocytes migration at site of inflammation. Leukocytes undergo a multi-step adhesion cascade, then polarize and move by diapedesis across the endothelium. (HEC, human endothelial cells).

Indeed, using a specific anti-CD157 agonistic (or blocking) mAb, we demonstrated that CD157 regulates neutrophil and monocytes adhesion to the apical surface of endothelium, but slows a prolonged and disoriented motility over the endothelial cell layer, which results in a clearly impaired ability to cross the endothelium [65]. These findings were confirmed by *in vivo* observations that neutrophils obtained from patients with paroxysmal nocturnal hemoglobinuria, an acquired genetic disease characterized by lack of GPI-anchored proteins, including CD157, are characterized by severe defects in adhesion, migration and diapedesis [66].

1.2.4 CD157 and interaction with the ECM proteins

So far, it has become clear that overexpression of CD157 induces a variety of cellular responses both in leukocytes and other cell types, apparently unrelated from its enzymatic functions [63]. However, for many years the efforts to unravel the non-enzymatic functions of CD157 have been hampered by the lack of a known biological ligand. The crucial role of CD157 in cell adhesion, migration and invasion and its functional partnership with integrins, suggested the existence of a direct interaction between CD157 and proteins composing the extracellular matrix (ECM) and fostered the hypothesis that the ligand of CD157 could be found in the extracellular matrix. These observations paved the way to discover the ability of CD157 to bind with high affinity to the heparin-binding domain comprised in selected ECM proteins, such as fibronectin, type I collagen, fibrinogen, and laminin-1 (but not vitronectin) [67]. The direct interaction of CD157 and ECM proteins proved instrumental to promote the concomitant localization of CD157 and integrins in lipid rafts and to deliver intracellular signals regulating cell adhesion and spreading through the activation of MAPK and PI3K pathways [62, 67].

Overall, experimental evidence suggested that binding of human CD157 to selected ECM proteins mediates many of its biological effects in different physiological and pathological contexts.

2. Aim of the work

Increasing evidence suggests that the dynamic interactions between AML cells and bone marrow microenvironment plays a critical role in the modulation of response of this disease to apoptosis and chemotherapy. Moreover, emerging evidence demonstrates that many anti-apoptotic signals, including BCL-2 and Mcl-1, are major causes of resistance to anticancer drugs in AML. Clinical trials with specific Bcl-2 inhibitors combined with toxic drugs provided promising results, and highlighted that AML is heterogeneous respect to anti-apoptotic protein dependence. Therefore, improved understanding of the biology of AML within the BM microenvironment, and the identification of predictive markers of response to specific anti-apoptotic combination regimens is a priority to design more effective therapy

CD157 is an adhesion molecule expressed on the surface of myeloid cells, BMSCs and selected epithelial cancer cells. It interacts with extracellular matrix proteins and regulates cell adhesion and migration via integrins signaling. Moreover, CD157 is involved in the protection of selected tumor cells from apoptosis and from platinum-induced cytotoxicity. CD157 is expressed in 97% of AML patients at time of diagnosis, although with remarkable inter-patient heterogeneity. In addition, CD157 is expressed in $CD34^{+}CD38^{-}$ leukemia-initiating cells that contribute to AML relapse. However, the functional role of CD157 in AML biology is largely unknown.

In this study, we describe the expression of CD157 in the leukemic cells, and its potential functional role in the pathogenesis of the disease, and address its potential involvement in the cross-talk between AML blasts and BM niche, by focusing on the following aims:

<u>Aim 1</u>

Analysis of CD157 expression on primary AML blasts, and evaluation of the biological significance of CD157 in the leukemic blasts *ex vivo* and *in vitro*.

<u>Aim 2</u>

Analysis of the role of CD157-mediated signaling in response to stress induced by nutrient deprivation or exposure to cytotoxic drugs, and identification of the mechanistic basis of the effects mediated by CD157 stimulation by using *in vitro* cell line models genetically engineered for CD157 expression.

Aim 3

Definition of the functional role of CD157 in the interaction between leukemic cells and bone marrow microenvironment and analysis of its contribution to the environment-mediated drug resistance *in vitro*.

* The majority of data presented in this thesis are published in:
Yakymiv Y., et al., *CD157 signaling promotes survival of acute myeloid leukemia cells and modulates sensitivity to cytarabine through regulation of anti-apoptotic Mcl-1*. Scientific Reports, 2021

3. Materials and Methods

3.1 Patients samples and isolation of human bone marrow stromal cells

Peripheral blood (n =11) or bone marrow (n = 8) samples from patients with newly diagnosed AML at diagnosis, were obtained from the A.O.U Città della Salute e della Scienza (Torino, Italy) and from the A.O.S. Croce e Carle (Cuneo, Italy) hospitals. The data were collected in accordance with the Declaration of Helsinki and good clinical practice guidelines. The study was approved by the local Institutional Review Board (Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino - A.O. Ordine Mauriziano - A.S.L. TO1 and A.O.S. Croce e Carle, Cuneo, Italy), and full written informed consent was obtained from all patients involved in the study. Peripheral blood mononuclear cells and bone marrow mononuclear cells were separated by standard Ficoll-Hypaque density-gradient centrifugation. Mononuclear cells were cultured in DMEM medium supplemented with 20% fetal calf serum (FCS), 2 mM L-glutamine and 100-unit penicillin, 100 μg/ml streptomycin. According to the number of cells obtained from each sample, AML cells were used immediately for *ex vivo* experiments, or frozen in FCS with 20% DMSO.

Human primary BMSCs were isolated from BM aspirates from AML patients. Mononuclear cells were purified by Ficoll-Hypaque density-gradient centrifugation, and re-suspended in DMEM with 20% FCS followed by plating at an initial density of $3x10^4$ cells/cm^2. After 3 days, the non-adherent cells were removed and monolayers of adherent cells were cultured for at least 10 days, until they reached confluence. The primary BMSCs were characterized by using anti-CD45-PE/Cy7, CD90-PE, CD105-FITC and CD157-APC antibodies by means of flow cytometry.

3.2 Cell lines

U937 and THP1 AML cell lines (from American Type Culture Collection, ATCC, Manassas, VA, USA) were grown in RPMI-1640 medium supplemented with 10% FCS; OCI-AML3 cells (kindly provided by G. Saglio, University of Torino, Italy) were cultured in DMEM medium supplemented with 20% FCS. The human BMSC line HS-5 was kindly provided by M. Fabbi (San Martino Hospital, Genova, Italy), and grown in DMEM medium with 10% FCS. All culture media were supplemented with 100-unit penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Morphology of cell lines was

monitored routinely, and mycoplasma contamination was excluded using a PCR-based assay. DNA fingerprinting analysis using 16 different, highly polymorphic short tandem repeat loci (PowerPlex 16 HS System, Promega) confirmed cell authenticity.

3.3 Antibodies and reagents

The SY11B5 anti-CD157 (IgG1) mouse monoclonal antibody (mAb) was produced in-house and affinity-purified on protein G. RF3 anti-CD157 (IgG1) mAb produced in mouse was purchased from MBL (Voden-Medical, Meda, MB, Italy). The isotype control IgG1 produced in mouse (mIgG, clone MG1-45) was purchased from Biolegend (Aurogene, Roma, Italy). Purified murine IgG Fc fragments were from Jackson ImmunoResearch (West Grove, PA). Antibodies to phospho-mTOR Ser-2448 (D9C2, #5536), phospho-p70S6K Thr-389 (108D2, #9234), phospho-AKT Ser-473 (D9E, #4060), phosphor-GSK-3β Ser-9 (D85E12, #5558), phosho-ERK1/2 Thr-202/Tyr-204 (D13.14.4E, #4370), phosho-S6 ribosomal protein Ser-235/236 (91B2, #4857), phosho-4E-BP1 Thr-37/46 (236B4, #2855), mTOR (L27D4, #4517), AKT (40D4, #2920), GSX3β (27C10, #9315) PARP-1 (46D11, #9532), Caspase-3 (D3R64, #14220), Caspase-9 (C9, #9508), Bcl-XL (54H6, #2764), Bcl-2 (D55G8, #4223), Bim (C34C5, #2933), Bax (D2E11, #5023), Bak (D4E4, #12105), Mcl-1 (D35A5, #5453), HRP-conjugated anti-mouse IgG1 (#7076) and HRP-conjugated anti-Rabbit IgG1 (#7074) were obtained from Cell Signaling Technology (Euroclone, Milan, Italy). Antibodies to β-actin (MAB-24008-HRP) and β-tubulin (MAB-80142-HRP) were purchased from Immunological Sciences (Roma, Italy). AraC was purchased from Sigma-Aldrich, S63845 and ABT-199/venetoclax inhibitors from Selleck Chemicals (Aurogene, Roma, Italy). Both ABT-199 and S63845 were dissolved in DMSO. Fibronectin from human plasma, Fibrinogen and Collagen type I were from Sigma-Aldrich (Milano, Italy). Vitronectin was from BD Biosciences. Calcein-AM was from Molecular Probes (ThermoFisher Scientific).

3.4 Immunofluorescence staining and flow cytometry analysis

CD157 surface expression on AML samples and cell lines was assessed by flow cytometry (BD FACS Canto, BD Biosciences) by staining cells (3×10^5/sample) with a PE-labeled mAb to CD157 (clone SY11B5, Invitrogen, Milan, Italy) for 20 min at 4°C.

Leukemic blasts were analyzed by multiparametric flow cytometry and gated based on CD45 expression (anti-CD45-APC/H7, BD Biosciences) and side scatter (CD45dim and SSClow). To further characterize blast subpopulations the following antibodies were used: CD33-FITC, CD34-PE/Cy7, CD64-PE, CD117-PE, CD123-PE/Cy7, HLA-DR-FITC (Biolegend). Specifically, this back-gating allowed us to identify leukemic myeloid blasts (CD33dim, CD64-, CD117+, CD123+, HLA-DR+) and leukemic monocytic populations (CD33+, CD64++, CD117low/neg, CD123+, HLA-DR++). Data were analyzed using FlowJo software Version 10.6 (BD Biosciences). CD157 relative Mean Fluorescence Intensity (MFI) was normalized to the MFI of lymphocytes, which are CD157 negative. CD157 MFI ratio was calculated according to the formula: (MFI of CD157 in blasts - MFI of CD157 in lymphocytes) / MFI of CD157 in lymphocytes.

3.5 CD157 gene knockdown by shRNA

CD157 expression in U937, THP1 and OCI-AML3 cell lines was silenced by lentiviral delivery of pLV-puro (Biosettia, San Diego, CA) encoding the BST-1 short-harpin RNA (shRNA) (target sequences: 5′-GAGTCAGACTGCTTGTATA-3′ (shCD157#1) and 5′-CCTGAGCGATGTTCTGTAT-3′ (shCD157#2) or a scrambled negative control (scrambled sequence: 5′-TTCTCCGAACGTGTCACGTT-3′), as previously described [68]. In details, cells were seeded and grown up to 50-60% of confluence, then the medium was replaced with RPMI or DMEM supplemented with 8µg/ml of Polybrene (Sigma-Aldrich). Cells were exposed to the lentiviral particles containing the BST-1 shRNA or a scrambled negative control at a multiplicity of infection (MOI) of 20. After 18h of incubation with the virus, the medium was replaced with RPMI or DMEM medium supplemented with 10% FCS and cells were further cultured for 24h. Transduced cells were then subjected to selection with 2 µg/ml Puromycine (Santa Cruz Biotechnologies) for 3 days.

3.6 CD157 gene transfection

HS-5 cells were transfected with 5 µg of linearized eukaryotic expression vector pcDNA3.1 (Invitrogen) containing the cDNA for full-length CD157 (BST-1; GenBank #NM_004334) or no insert (mock). Viromer red was used for all transfections. Stable

clones were selected in cell culture medium containing 2 μg/ml Puromycine (Santa Cruz Biotechnologies) for 3 days.

3.7 RNA extraction and RT-PCR

Total RNA was extracted from transduced AML cell lines using TRI reagent (Sigma-Aldrich), and quality and quantity checked with NanoDrop (ND-100, Thermo Fisher Scientific, Monza, Italy). 2 μg of RNA were reverse transcribed with the M-MLV Reverse Transcriptase (Invitrogen) and Oligo-dT primers (Sigma-Aldrich), as previously described [68]. PCR products were analyzed by electrophoresis on 1% agarose gel stained with Midori Green Advance DNA Stain (NIPPON Genetics Europe, Düren, Austria).

3.8 Antibody-mediated CD157 stimulation

In selected experiments, primary AML cells (5×10^5/ml) or cell lines (4×10^5/ml) were treated for 24 h (unless otherwise indicated) at 37°C in standard culture medium or in serum-free conditions, as indicated, with affinity purified SY11B5 anti-CD157 mAb (10 μg/ml), or with an isotype-matched mIgG (10 μg/ml), as control. Then, cells were washed once in RPMI 1640 medium and processed according to the indicated assay.

3.9 Flow cytometry analysis of apoptosis

Apoptosis was determined by flow cytometry measurement of phosphatidylserine exposure using Annexin V-FITC. Briefly, AML cells were treated with the indicated stimulus or drug for 24 h, washed once in RPMI 1640 medium, and then incubated with 100 μl of Annexin V binding buffer containing 2 μl of Annexin V-FITC and 2 μl of Propidium Iodide (PI) (Kit #IK-90314, Immunological Sciences) for 20 min at room temperature in the dark. Annexin V fluorescence was determined with a BD FACS Canto (BD Biosciences) flow cytometer and the membrane integrity of cells was simultaneously assessed by PI exclusion method. Flow cytometry analysis was performed by FlowJo software Version 10.6 (BD Bioscience). At least three independent experiments were performed with AML cell lines. Patient samples were chosen on the basis of availability of adequate cell number and experiments were performed once.

3.10 PrestoBlue viability assay

Primary AML cells (6×10^4/well) or AML cell lines (5×10^4/well) were seeded in 96-well plates in 100 µl of culture medium supplemented with FCS and treated as indicated. Drugs at the indicated dilutions were added to each well. At each time point, 10 µl/well of PrestoBlue reagent (Thermo Fisher Scientific) was added for 1 h at 37 °C, then fluorescence was measured by using the Infinite M200 Pro Microplate Reader (Tecan Italia, Cernusco sul Naviglio, MI, Italy) with excitation/emission wavelengths set at 560/590 nm. Cell viability was determined by comparing the fluorescence values before and after treatment and normalizing the data to controls. Half-maximal effective concentration (EC_{50}) values were calculated by non-linear regression algorithms and a four-parameter logistic model with log-transformed data using GraphPad Prism 7 software.

3.11 Western blot analysis and immunoprecipitation

Cells were washed and harvested in ice-cold phosphate-buffered saline and subsequently lysed in extraction buffer (50 mM Tris HCl pH7.4, 150 mM NaCl, 1% Triton-X 100, 0.5% Sodium Deoxycholate, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 0.1% SDS) supplemented with 1 mM Na3VO4 and protease inhibitor cocktail. Protein concentration was determined using the Bradford assay (BioRad Laboratories, Segrate, MI, Italy). Equal amounts of proteins were separated by an Any-kD Critereon TGX Stain-free PreCast gel (BioRad) and transferred to nitrocellulose membranes (Trans-blot Turbo Transfer pack, BioRad). After blocking with 2% BSA for 1 h, membranes were cut to minimize antibody use and each strip was separately probed with the indicated primary antibody, followed by the appropriate HRP-conjugated secondary antibody. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL, BioRad), images were captured with a ChemiDoc XRS+ System. Band densitometry analysis was performed with Image Lab Software (BioRad).

For immunoprecipitation, after the indicated treatments, cell extracts were obtained using lysis buffer (containing 50 mM Tris HCl pH7.4, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate) supplemented with 5 mM EDTA, 10 mM NaF, 1 mM Na3VO4, 1 mM DTT and protease inhibitor cocktail. An equivalent of 1 mg/sample of protein extracts

was incubated overnight at 4°C with anti-Mcl-1 (D2W9E, #94296) or anti-Bim (C34C5, #2933) antibodies (Cell Signaling, Euroclone, Milan, Italy), and subjected to immunoprecipitation with 20 µl of anti-rabbit IgG-coated Sepharose beads (Cell Signaling) for 3 h at 4 °C. Proteins were eluted by boiling samples for 5 min at 95 °C with 3× SDS reducing sample buffer and then analyzed by Western blotting. Equal amounts (20 µg) of lysate was prepared as described above, and used as whole-cell lysate control.

3.12 Cell adhesion assays

HS-5 cells were seeded at 5×10^4 cells in 96-well black plate and incubated overnight in complete medium. AML cells were labelled with 5 µM calcein-AM for 30 min at 37°C, washed and then seeded in serum-free medium onto 96-well black plates coated with the indicated extracellular matrix proteins (all at 10 µg/mL) or on HS-5 cell monolayers. Cell were incubated at 37°C for 1 h, and subsequently washed twice, to remove the non-adherent cells. Relative fluorescence intensity (RFI) of calcein-AM was measured at 515 nm using the Infinite M200 Pro Microplate Reader (Tecan). Results show the ratio between the RFI measured after washing and the RFI measured before washing [(RFI after /RFI pre-wash wash) ×100]. Where indicated, cells were pre-treated for 20 min at RT with anti-CD157 (SY11B5), anti-HLA Class I, anti-β1 integrin, anti-α4 integrin and anti-α5 integrin mAbs (all at 10 µg/ml) for 20 min, then cells were seeded on FN-coated plates (10µg/ml) and incubated for 1 h at 37°C.

3.13 Co-culture assay

HS-5 cells were seeded at 7×10^4 in 48-well plates and incubated overnight in complete medium. Next, 1.5×10^5 AML cells were cultured alone or onto HS-5 monolayers in the appropriate medium supplemented with 5% FCS. After 30 min of co-culture, cells were exposed to 10 µM AraC for 48 h. Cells were collected and stained with CD90-APC to discriminate HS-5 (CD90-positive) from AML cells (CD90-negative). After 30 min at 4°C, cells were washed and stained for Annexin V/PI or for cell cycle analysis.

3.14 Cell cycle analysis

AML cells were harvested and washed with washing buffer (PBS, 0.5% BSA, 0.5 mM EDTA). Then, cells were fixed in 70% ice-cold ethanol overnight at -20°C, washed twice and RNA was digested with RNase A (Sigma-Aldrich) for 30 min at 37°C. Then, cells were stained with 20 μg/ml of PI. After 10 min of incubation at 4°C, DNA content was determined by flow cytometry.

3.15 Statistical analysis

Unless otherwise indicated, results of *in vitro* experiments are expressed as means ± SEM of at least three independent experiments. Comparisons among multiple groups were performed with both one-way and two-way ANOVA models. Differences between two groups were analyzed using unpaired two-tailed Student's t-test. When the data were not normally distributed, the comparison of variables was performed with a Wilcoxon signed rank test for paired data or with a Mann-Whitney test for unpaired data. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). For all analyses, p values <0.05 were considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus control, and ns for not significant).

4. Results

4.1 CD157 expression in primary AML blasts

In the present study a comprehensive analysis of CD157 expression in primary AML blasts has been performed. In collaboration with the Department of Hematology at A.O.U. Città della Salute e della Scienza of Torino and A.O.S Croce e Carle of Cuneo hospitals, we collected 19 samples of bone marrow (BM, n=8) or peripheral blood (PB, n=11) of newly diagnosed treatment naïve AML patients. Our cohort included 5 males and 14 females with the median age of 59.3 years (range, 25-86 years). The clinical characteristics of the patients included in this study are summarized in Table 4.

#	Age	Sex	FAB	WHO classification	FLT3 ITD	NPM1	Type of sample	Blasts (%)	Blasts $CD157^{low}$ (%)	Blasts $CD157^{dim}$ (%)	Blasts $CD157^{high}$ (%)
1	57	F	M5b	Acute monocytic leukemia	wt	mut	PB	44	4	40	
2	50	M	M4	Acute myelomonocytic leukemia	mut	wt	BM	92		92	
3	38	M	M4	Acute myelomonocytic leukemia	wt	wt	BM	44		29	15
4	51	F	M4	Acute myelomonocytic leukemia	mut	mut	PB	93		93	
5	37	F	M4	Acute myelomonocytic leukemia	wt	wt	BM	85	32		53
6	52	F	NA	AML with recurrent genetic alterations	wt	wt	BM	43	34	9	
7	25	M	M4	Acute myelomonocytic leukemia	wt	wt	PB	36		27	9
8	50	F	M4	Acute myelomonocytic leukemia	mut	mut	BM	64		38	26
9	66	F	NA	AML with MDS	wt	wt	BM	53	53		
10	77	F	M4	Acute myelomonocytic leukemia	wt	wt	BM	85		85	
11	72	F	NA	NA	NA	NA	BM	79	55	24	
12	72	F	M2	AML with maturation	wt	mut	PB	70	70		
13	55	M	M1	AML with minimal maturation	wt	wt	PB	78	78		
14	68	F	M5	Acute monocytic leukemia	wt	mut	PB	47			47
15	47	F	M2	AML with maturation	wt	wt	PB	20	20		
16	77	F	M4	Acute myelomonocytic leukemia	wt	wt	PB	23	2		21

17	53	F	M4	Acute myelomonocytic leukemia	wt	wt	PB	76	10		66
18	69	F	M2	AML with maturation	mut	mut	PB	77	22	55	
19	86	M	NA	Secondary AML	wt	wt	PB	97	61	36	

Table 4. Clinical characteristics of AML patients included in this study. Abbreviations: FAB = French–American–British classification; WHO = World Health Organization classification; F = female; M = male; MDS = myelodysplastic syndrome; FLT3 = fms-like tyrosine kinase 3 gene; ITD = internal tandem duplication; NPM1 = nucleophosmin 1 gene; mut = mutated; wt = wild type; NA = not available; PB = peripheral blood; BM = bone marrow. CD157 Mean Fluorescence Intensity (MFI) ratio was calculated according to the formula (MFI of CD157 on blasts - MFI of CD157 on lymphocytes) / MFI of CD157 on lymphocytes. CD157low = MFI ratio <10; CD157dim = MFI ratio ≥10 <50; CD157high = MFI ratio ≥50.

First, AML blasts were compared for the expression of conventional surface markers used to define myeloblasts maturation [69], and the expression of CD157 on the blasts membrane was assessed by mean of multiparametric flow cytometry analysis. The gating strategy for the identification of AML blast population is shown in Figure 5.

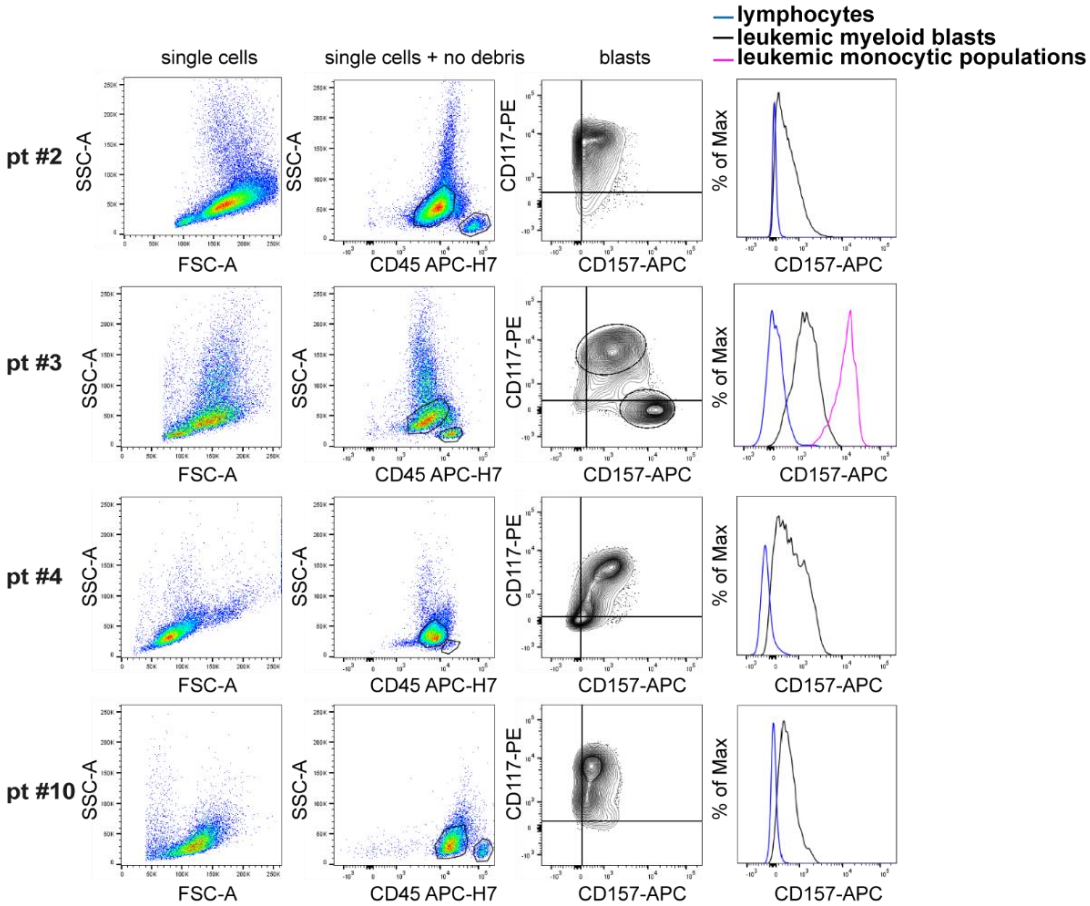


Figure 5. Gating scheme of flow cytometric analysis of CD157 expression in AML blasts. After excluding doublets and debris, blasts and lymphocytes were selected based on their SSC and CD45 expression profiles. Representative FACS plots of CD157 expression in four samples from patients with AML used for functional experiments are shown. CD117 expression was used to further characterize blast populations. SSC, side scatter; FSC, forward scatter.

In details, after excluding doublets and debris, lymphocytes and blasts were identified as $CD45^{dim}$ and SSC^{low} and blasts were further distinguished in myeloblasts ($CD33^{dim}$, CD64-, CD117+, CD123+, HLA-DR+) and immature monocytic populations (CD33+, CD64++, $CD117^{low/neg}$, CD123+, HLA-DR++). Thereafter, the back-gating of these two blast populations allowed for further analysis of CD157 expression. CD157 was expressed on all samples so far analysed, although at variable levels (Figure 6A). Notable, the expression of CD157 in myeloblast ($CD117^{pos}$), representing an early differentiation stage, was intermediate, whereas, immature monocytic blasts ($CD117^{neg}$), expressed significantly higher levels of CD157 (Figure 6A, B). The results of this multiparametric analysis suggested that there is an inverse correlation between the expression of CD157 and CD117 in leukemic blasts, confirming that CD157 expression in AML blasts progressively increases with myeloid cell maturation.

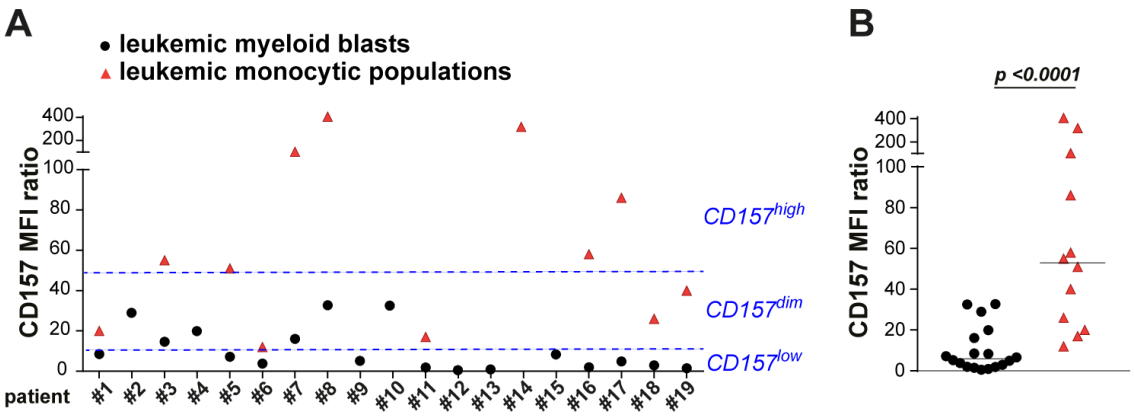


Figure 6. CD157 MFI distribution in a group of AML patients. A) The y-axis shows the mean fluorescence intensity (MFI) of CD157 expression on leukemic myeloid blasts (black dots) and leukemic monocytic populations (red triangles) in each patient. Blue dotted lines indicate the CD157 MFI ratio used as cutoff: $CD157^{low}$ = MFI ratio <10; $CD157^{dim}$ = MFI ratio ≥10 <50; CD157 ^{high} = MFI ratio ≥50. B) Comparison of CD157 expression in leukemic myeloid blasts versus leukemic monocytic population ($p < 0.0001$, Mann-Whitney test).

4.2 The biological role of CD157 in the behavior of AML blasts

4.2.1 Role of CD157 in AML blasts survival *ex vivo*

In vitro culture is widely used for characterization of primary AML blast population, but even in optimized standard culture conditions, primary AML cells undergo spontaneous or stress-induced apoptosis, due to absence of soluble factors and cell-cell contacts [70]. To evaluate the functional role of CD157 in the biology of AML blasts, we used mononuclear cells obtained from 19 newly diagnosed AML patients. To test whether CD157 could affect AML cells viability *ex vivo*, cells were maintained for 24 h in DMEM culture medium supplemented with 20% FCS in the presence of an agonistic anti-CD157 mAb (SY11B5) or an isotype-matched control mAb (mIgG). The apoptosis was then analyzed by Annexin V/PI staining and flow cytometry analysis. After 24 h, AML cells showed variable levels of apoptosis ranging from 15.6 to 92.5%. However, the percentage of apoptotic cells decreased after CD157 mAb treatment with respect to untreated or mIgG isotype-matched treated controls, for every sample tested (Figure 7A, B).

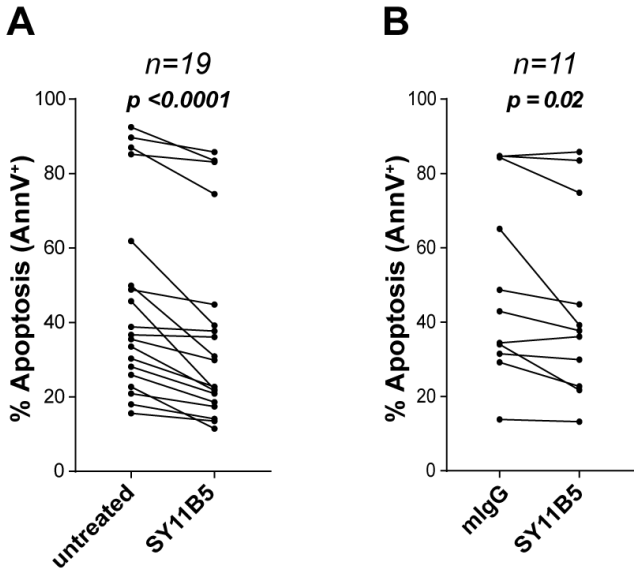


Figure 7. Impact of CD157 ligation on primary AML cells viability. Annexin V/PI staining and flow cytometry analysis showing the percentage of apoptotic cells in samples maintained in standard culture conditions and treated for 24 h with **A, B)** SY11B5 anti-CD157 mAb (10µg/ml) or **B)** mIgG isotype-matched control mAb (10µg/ml). Each dot represents a single patient. ($p < 0.0001$ and $p = 0.02$, Wilcoxon's signed-rank test).

Next, the correlation between CD157 expression and *ex vivo* survival of AML blasts was evaluated. Among the cohort of patients used in this study, five samples showed >50% apoptosis *ex vivo* had low expression of CD157. However, the correlation between CD157 expression and blasts survival was not statistically significant, at least in the small number of samples analyzed (Figure 8).

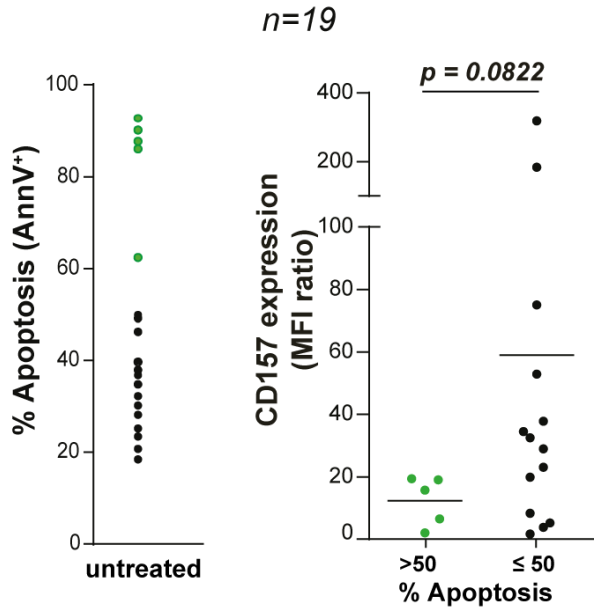


Figure 8. Impact of CD157 expression levels on AML cell survival *ex vivo*. Mononuclear cells from AML patients (n = 19) were maintained for 24 h under standard culture conditions (untreated), then the percentage of apoptotic cells was determined by Annexin V/PI staining and flow cytometry analys (left panel). Each dot represents a single patient. Green dots represent samples with >50% of apoptotic cells. Right panel shows CD157 MFI ratio in AML samples with high (>50%) and low (<50%) spontaneous apoptosis *ex vivo*. (p = 0.0822, Mann-Whitney test).

The above experiments were performed using the whole mononuclear cell fraction from blood, including normal lymphocytes and myeloid cells, beside blast population. To determine if the pro-survival effect mediated by CD157 was specific to leukemic blasts, FACS analysis of forward scatter (FSC) and side scatter (SSC) parameters were carried out. As shown in Figure 9, after 24 h of *ex vivo* culture in the presence or absence of anti-CD157 mAb or control mIgG, the percentage of viable blasts remained almost unchanged in the CD157 antibody-treated sample (t = 0: untreated 42.6% *versus* t = 24 h: SY11B5 40.1%), while it was notably reduced in untreated and mIgG-treated samples (t = 0: untreated 42.6% *versus* t =24 h: untreated 27.2% and mIgG 29.6%). Notably, the

percentage of lymphocytes remained unchanged upon treatment, indicating that the pro-survival effect elicited by CD157 ligation was restricted to leukemic cells.

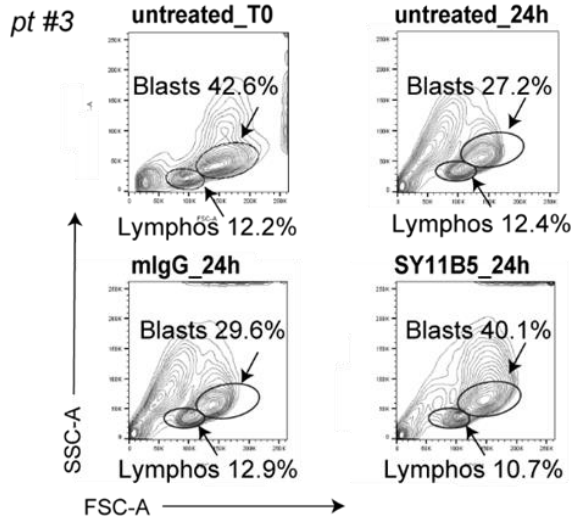


Figure 9. Effect of CD157 ligation in mononuclear cells from patient with AML. Density plots showing the distribution of each population composing the mononuclear cell fraction derived from a sample of BM from a representative AML patient at time zero (T0), after 24 h in standard medium (untreated), in the presence of mIgG or SY11B5 anti-CD157 mAb. Lymphocytes (FSC^{low}/SSC^{low}) and blasts (FSC^{dim}/SSC^{dim}) were gated according to morphological parameters. Percentage indicates the frequency of each population compared to total acquired events.

In addition, the CD157-mediated pro-survival effect was dose-dependent (Figure 10A) and persisted over time to at least 72 h (Figure 10B), indicating specificity.

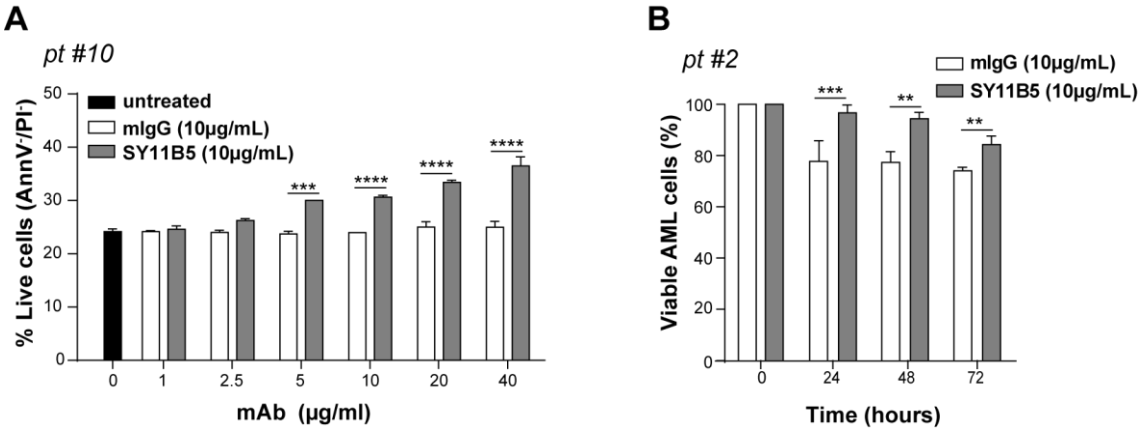


Figure 10. Pro-survival effect of SY11B5 anti-CD157 mAb in primary AML cells. A) Annexin V/PI staining analysis showing the percentage of $AnnV^-/PI^-$ cells exposed to increasing concentrations of SY11B5 anti-CD157 mAb or mIgG for 24 h. A representative experiment performed with AML cells from patient #10 is shown. **B)** PrestoBlue assay showing the % of viable cells at each time point compared to time zero. A representative graph performed in quadruplicate is shown. (**p <0.01, ***p <0.001, ****p <0.0001, two-way ANOVA with Sidak's multiple comparison test).

4.2.2 CD157 ligation triggers the activation of intracellular signals and regulates apoptosis in AML blasts

To better understand the molecular mechanism through which CD157 promoted AML cells survival *ex vivo*, we analysed the intracellular signals mediated by CD157. The focus was concentrated on PI3K/AKT/mTOR and MAPK/ERK signal transduction pathways, known to be activated upon CD157 cross-linking by means of specific mAbs in monocytes [62], and are frequently deregulated in AML [71, 72].

Ligation of CD157 by CD157-targeted antibody for 24 h resulted in a remarkable increase in phosphorylation of mTOR and its downstream substrates p70S6K, pS6 ribosomal protein and 4E-BP1; ERK and AKT on Ser473, leading to the inactivating phosphorylation of GSK-3β (Figure 11A). GSK-3β, a major AKT target, is implicated in the regulation of many pathways, including apoptosis [73], and is associated with poor survival outcome in AML patients [74]. These notions led us to investigate if CD157 stimulation could modulate apoptosis, by examining the expression of Bcl-2-family proteins. Western blot analysis of primary AML cells treated with anti-CD157 mAb showed a remarkable increase of Mcl-1 and Bcl-XL anti-apoptotic proteins, whereas Bcl-2 protein levels were only marginally affected. Moreover, the pro-apoptotic protein Bax was clearly downregulated upon CD157 antibody binding, accompanied by reduced proteolytic cleavage of Caspase-3 and its substrate PARP-1, which are considered to be hallmarks of apoptosis (Figure 11B). Collectively, these observations highlighted that CD157-mediated signaling improves survival of AML blasts through the activation of specific anti-apoptotic signals.

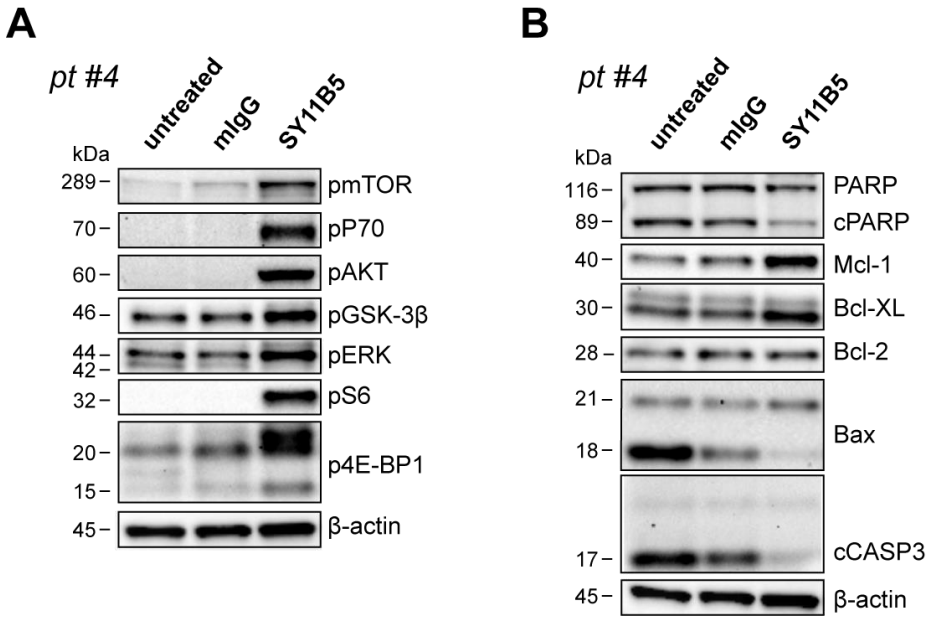


Figure 11. CD157 ligation promotes the activation of pro-survival PI3K/AKT/mTOR pathway and Bcl-2 anti-apoptotic pathway. Western blot analysis following CD157 stimulation for 24 h with SY11B5 anti-CD157 mAb or mIgG (both at 10 µg/ml) showing phosphorylation levels of the indicated proteins of **A)** PI3K/AKT/mTOR pathway and **B)** Bcl-2 apoptotic pathway. A representative Western blot out of four patients analysed is shown. β-actin was used as loading control.

4.2.3 CD157 modulates AraC-mediated cell death in primary AML cells

After having shown that CD157 ligation induces pro-survival signals in AML cells, a possible influence of CD157 triggered mediated signaling on the sensitivity of tumor cells to cytotoxic drug AraC, was investigated. To address this issue, eight BM samples from AML patients were pretreated with SY11B5 anti-CD157 mAb or mIgG immediately before exposure to 10 µM of AraC. After 24 h, apoptosis was assessed by flow cytometry. Ligation of CD157 by SY11B5 mAb significantly reduced the number of apoptotic cells, compared to the corresponding controls treated with mIgG (Figure 12), indicating that CD157 signaling could interfere with AraC-induced apoptosis in primary AML cells.

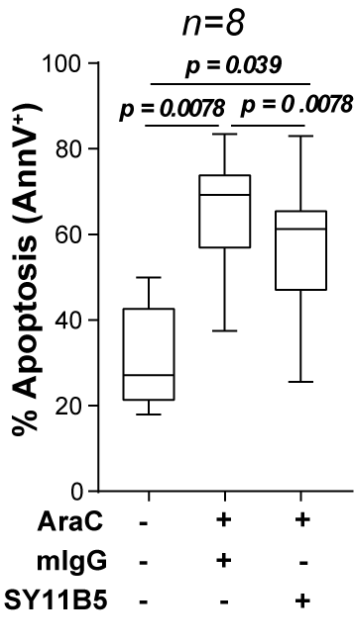


Figure 12. Effect of the CD157 ligation in primary AML blasts exposed to AraC treatment. Annexin V/PI staining and flow cytometry analysis of eight AML samples treated with 10 µM AraC in the presence of anti-CD157 mAb or mIgG (both at 10 µg/ml) for 24 h. The percentage of apoptotic cells (AnnV+) is shown. (p values are indicated, Wilcoxon's signed-rank test).

4.3 Functional role of CD157 in AML cell lines

4.3.1 CD157 expression in AML cell lines used as *in vitro* models

To analyse more in detail the role of CD157 in the behavior of AML cells, the study was extended to U937, THP1 and OCI-AML3 human AML cell lines, all expressing variable levels of CD157 protein (Figure 13), and subsequently used as *in vitro* models.

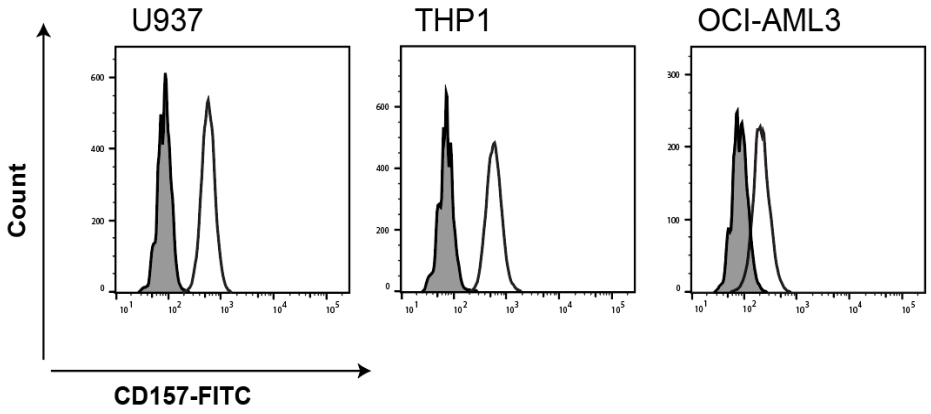


Figure 13. CD157 expression in AML cell lines. Flow cytometry analysis of the expression of CD157 protein in U937, THP1 and OCI-AML3 cells. Cells were stained with the SY11B5 anti-CD157 mAb (white peaks) or with an isotype-matched mAb (grey peaks).

4.3.2 Effect of CD157 engagement in AML cells

To determine the propensity of U937, THP1 and OCI-AML3 cells to apoptosis under stressful conditions, AML cell lines were grown in culture medium without FCS for 24 h. Cell viability resulted to be significantly affected in OCI-AML3 cells, whereas, U937 and THP1 cells were only marginally affected (Figure 14).

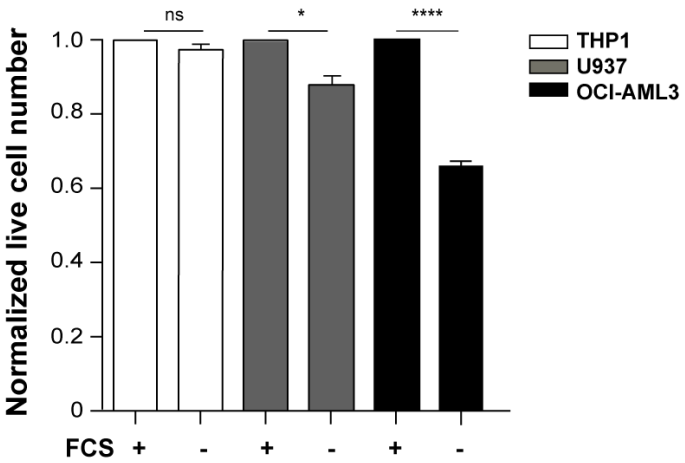


Figure 14. Effect of serum starvation on AML cell viability. Annexin V/PI analysis of THP1, U937, OCI-AML3 cells cultured in the absence of FCS for 24 h. Cell viability is expressed as fold change of viable cells compared to cells maintained in standard culture conditions, and are the mean ± SEM of tree independent experiments. (ns = not significant, *p <0.05, ****p <0.0001, Student's *t*-test).

To reproduce the pro-survival effect mediated by CD157 ligation in primary AML cells, OCI-AML3 cells were treated with anti-CD157 mAb or mIgG control mAb for 24 h, following overnight starvation. Results showed a significantly improved cell survival after treatment with SY11B5 mAb (Figure 15A). Similar results were obtained by pretreating OCI-AML3 cells with saturating concentrations of murine IgG Fc fragments, thus excluding the potential interference of Fc receptor-mediated effect (Figure 15B).

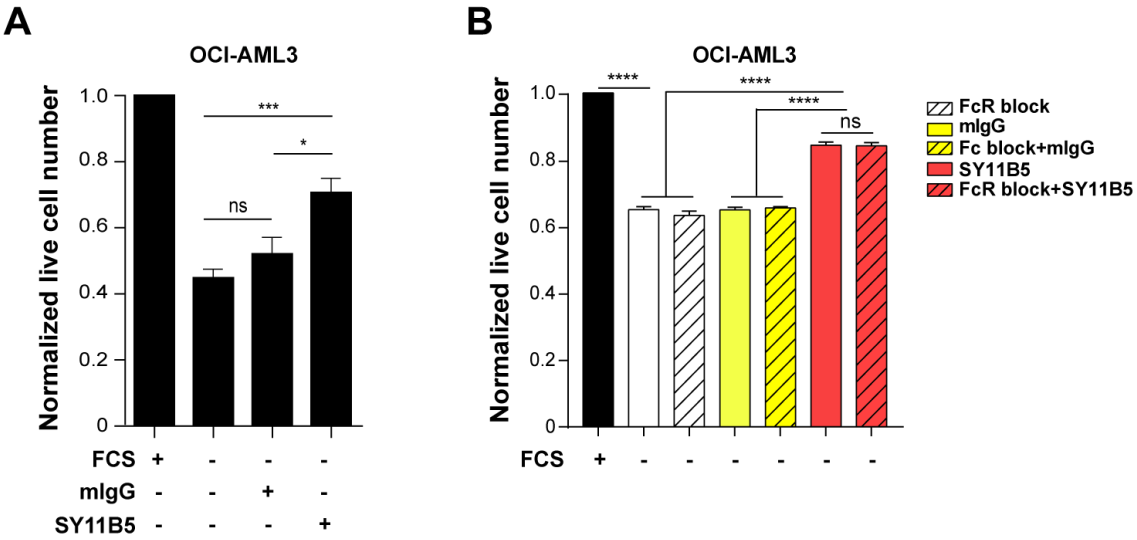


Figure 15. CD157 ligation promotes survival in OCI-AML3 cells. A) Annexin V/PI analysis of OCI-AML3 cells cultured overnight in the absence of FCS, and then treated with SY11B5 mAb or mIgG (both at 10µg/ml) for 24 h. **B)** Annexin V/PI analysis of OCI-AML3 cells treated with SY11B5 mAb or mIgG (both at 10µg/ml) for 24 h, after overnight starvation, in the presence or absence of mIgG Fc fragment (100µg/ml). Cell viability is expressed as fold change of viable cells compared to cells maintained in standard culture conditions, and are the mean ± SEM of tree independent experiments. (ns = not significant, *p <0.05, ***p <0.001, ****p <0.0001, one-way ANOVA test).

4.3.3 AML cell lines engineered for the CD157 expression

To combine the antibody-mediated approach with genetic manipulation of CD157 expression in AML cells, we generated stable CD157 knockdown versions from the parental cell lines using lentiviral vectors expressing two shCD157 (shCD157#1 and shCD157#2) or a scramble control sequence that conserved endogenous expression of CD157 (CD157high cells) (Figure 16A, B). For further analysis, we selected cells with the lowest expression of CD157 (CD157low cells) in order to compare CD157high versus CD157low AML cells.

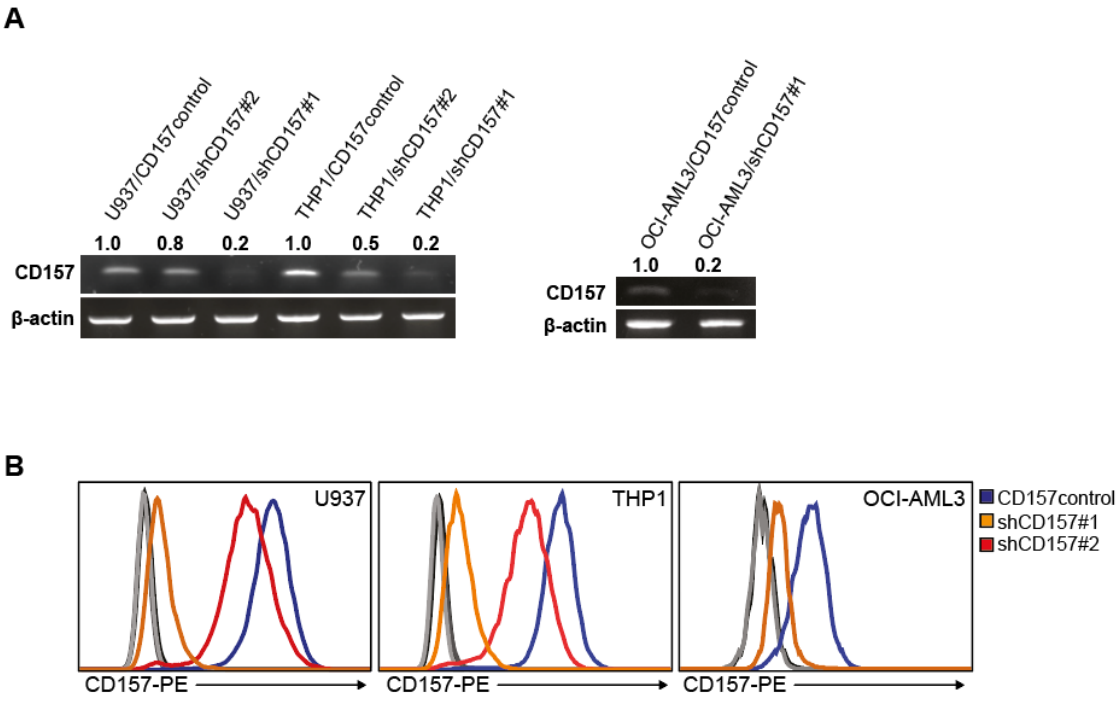


Figure 16. CD157 expression in AML cell lines. A) RT-PCR analysis of U937, THP1 and OCI-AML3 cell lines stably transduced with a CD157-specific shRNA (shCD157#1 or shCD157#2) or a scrambled shRNA control (CD157 control). β-actin was used as controls. **B)** Flow cytometry analysis of CD157 protein expression levels by staining cells with the anti-CD157 PE-conjugated mAb. An isotype matched mAb (gray peaks) was used as control.

4.3.4 Effect of CD157 knockdown on AML cells proliferation and survival

In order to evaluate the effect induced by the loss of CD157, we first determined whether AML cell proliferation was affected *in vitro*. Results showed that CD157 knockdown did not affect the AML cell lines proliferation ability, when cultured in standard culture conditions (Figure 17A). However, when cultured in medium without FCS for 24 h, the CD157low cells showed significantly lower number of viable cells, as compared to the CD157high cells cultured in the same experimental conditions (Figure 17B).

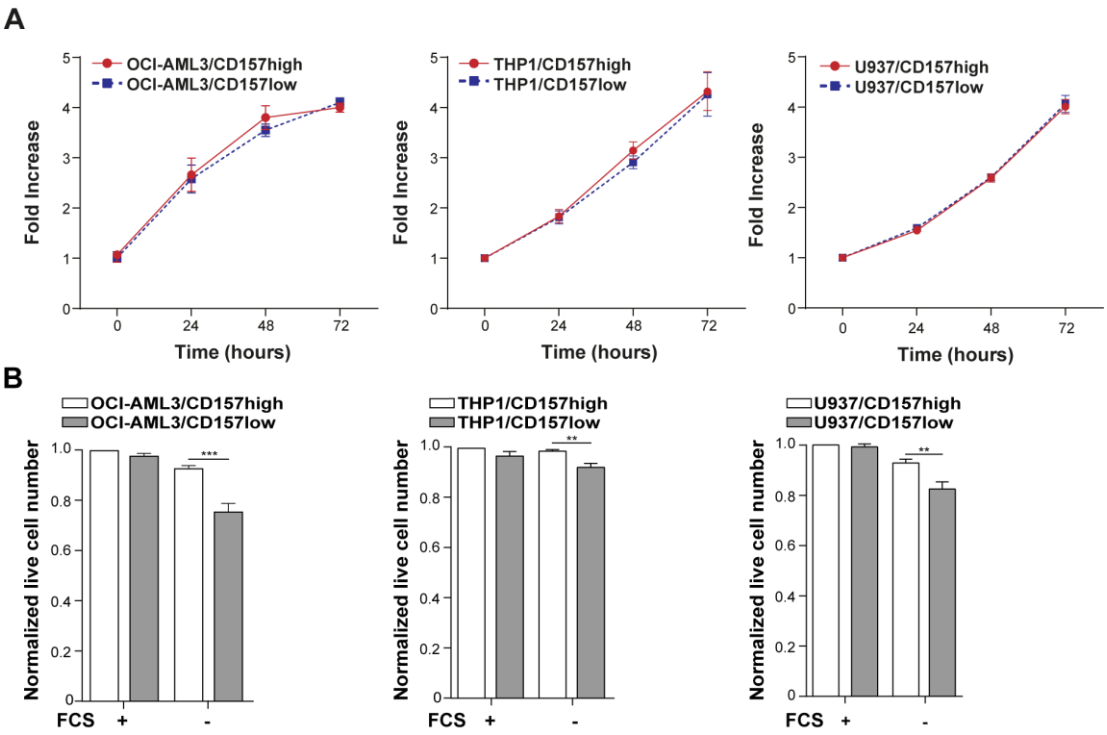


Figure 17. Effect of CD157 knockdown on AML cell lines proliferation and sensitivity to nutrient deprivation. A) PrestoBlue proliferation assay of OCI-AML3, THP1 and U937 AML cell lines engineered for the expression of CD157. Results are expressed as fold increase compared to time zero and are the mean ± SEM of three independent experiments performed in quadruplicate. **B)** Annexin V/PI analysis of engineered OCI-AML3, THP1 and U937 AML cell lines cultured in the absence of FCS for 24 h. Cell viability is expressed as fold change of viable cells compared to cells maintained in standard culture conditions, and are the mean ± SEM of tree independent experiments. (**p <0.01, ***p <0.001, one-way ANOVA test).

To decipher the molecular pathway through which CD157 mediates its pro-survival effect in AML cell lines, we focused on selected intracellular signaling pathways, known to play a role in cell survival. We previously demonstrated that CD157 interaction with

fibronectin, its natural ligand, activates the PI3K/AKT/mTOR signaling pathway [67]. To evaluate the activation of the above mentioned pathway, we selected the engineered versions of THP1 and U937 cell lines (OCI-AML3 were excluded as the parental line has the lower CD157 expression) and treated them with FCS as a source of ECM proteins. In details, after overnight starvation, cells were maintained in following conditions: i) standard culture, ii) without FCS, iii) with 10% FCS for 10 min or, iv) with 10% FCS for 2 h. Western blot analysis showed that the addition of FCS to CD157high THP1 and U937 cells resulted in a more robust phosphorylation of AKT, GSK-3β kinase and S6 ribosomal protein, and increased the expression of Mcl-1 (especially after 10 min of exposure to FCS), as compared to their CD157low counterparts (Figure 18A, B). Together, these data indicate that stimulation of CD157 significantly activated the PI3K/AKT/mTOR pathway while inactivated GSK-3β kinase, resulting in the upregulation of the Mcl-1 protein.

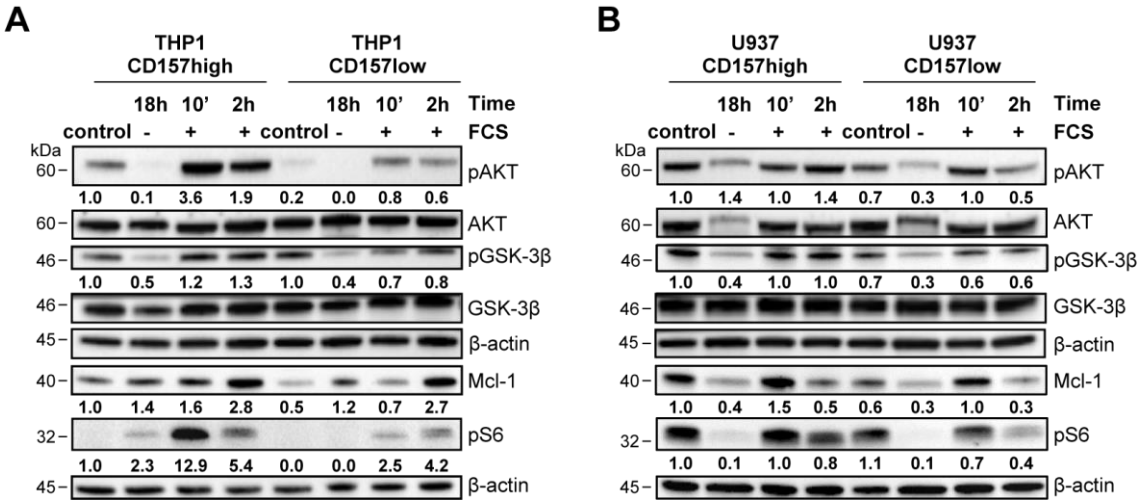


Figure 18. Intracellular pathway triggered by CD157 stimulation by FCS. CD157high and CD157low THP1 **A)** and U937 **B)** cells were maintained for 18 h in FCS-free medium, then stimulated with FCS for 10 min and 2 h. Whole cell lysates were subjected to Western blotting and probed with the indicated antibodies. β-actin was used as loading control. Numbers below blots indicate fold change in the expression of each protein relative to untreated CD157high cells, normalized to the corresponding β-actin (pS6, Mcl-1) or total protein (pAKT, pGSK-3β).

4.3.5 Effect of CD157 knockdown in AML cell lines sensitivity to AraC

Having established that CD157 might have role in modulating AML cells apoptotic pathway, we then analysed the impact of CD157 signaling in response of AML cells to cytotoxic agents, such as Cytarabine (AraC). First, we tested the viability of parental

THP1, OCI-AML3 and U937 AML cell lines exposed to increasing concentrations of AraC. After 24 h of treatment, U937 proved to be the only cell line sensitive to AraC, whereas THP1 and OCI-AML3 cells resulted resistant (Figure 19), probably due to different pharmacokinetics between cell lines and their reduced cytidine deaminase activity [75]. Accordingly, the following experiments involving AraC were performed using the U937 cell line.

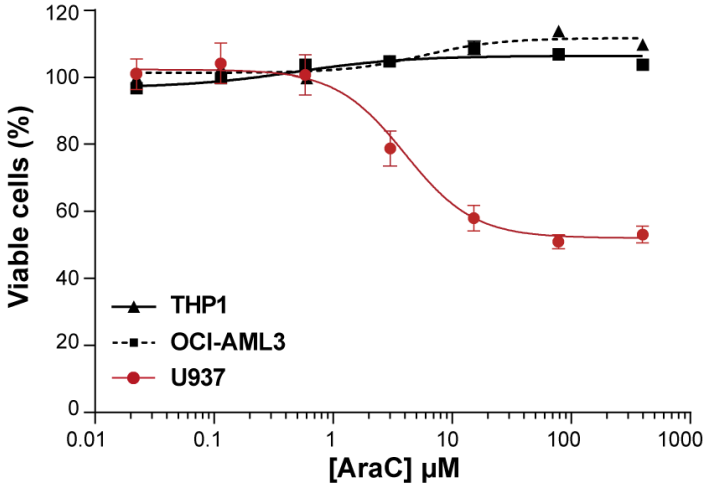
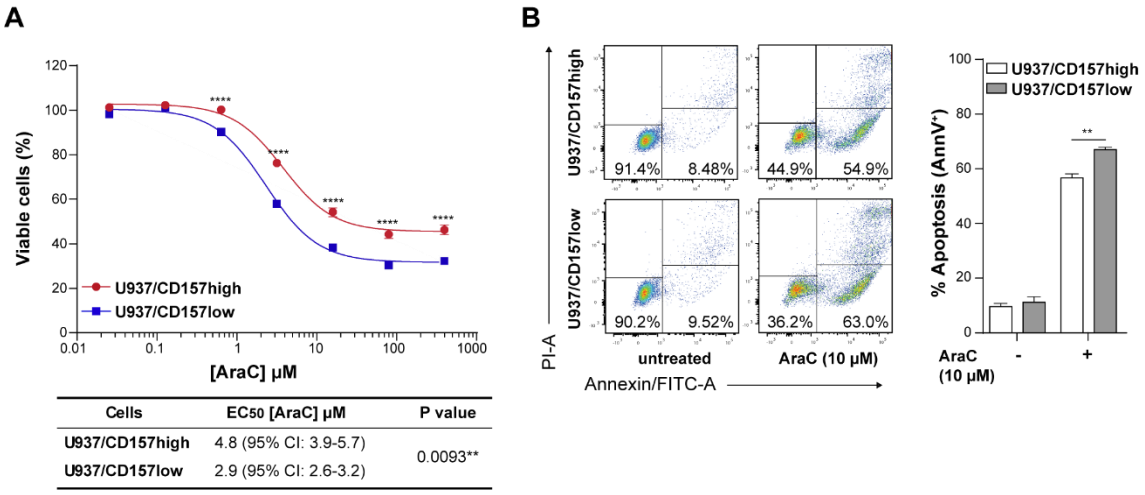


Figure 19. Sensitivity of AML cell lines to AraC. PrestoBlue assay showing the viability of THP1, OCI-AML3 and U937 cells after treatment with increasing concentrations of AraC for 24 h. Results are expressed as mean ± SEM of three independent experiments performed in quadruplicate.

Same experiments were performed in CD157high and CD157low U937 cells following treatment with increasing concentrations of AraC, resulting in significantly higher sensitivity of U937/CD157low than CD157high cells (Figure 20A). Indeed, the half-maximal effective concentration (EC_{50}) of AraC decreased from 4.8 µM in CD157high cells to 2.9 µM in CD157low cells (p=0.0093). Moreover, following treatment with 10 µM of AraC for 24 h, the percentage of apoptotic U937/CD157low cells was significantly higher compared to the CD157high version (66.0% ± 2.9 *versus* 55.9% ± 3.1, respectively) (Figure 20B).



Cells	EC50 [AraC] µM	P value
U937/CD157high	4.8 (95% CI: 3.9-5.7)	0.0093**
U937/CD157low	2.9 (95% CI: 2.6-3.2)	

Figure 20. Effect of CD157 knockdown on U937 cell sensitivity to AraC-induced apoptosis. **A)** PrestoBlue assay showing the viability of CD157high (red line) versus CD157low (blue line) U937 cells after treatment with increasing concentrations of AraC for 24 h. Results are expressed as mean ± SEM of four independent experiments performed in quadruplicate. (****p <0.0001, Student's t-test). B) AnnexinV/PI analysis showing representative dot plots (left) and histograms (right) of CD157high and CD157low U937 cells treated with AraC for 24 h. Histograms show the mean ± SEM of eight independent experiments. (**p <0.01, two-way ANOVA test).

We next investigated if CD157 signaling could decrease the sensitivity of U937 cells to AraC by modulating apoptosis. Treatment of U937 cells with AraC is known to induce apoptosis by Mcl-1 degradation and Bax cleavage with generation of a pro-apoptotic fragment of 18-kDa [76]. Western blot analysis showed that U937/CD157high cells expressed higher levels of Mcl-1 protein than CD157low cells, which was only partly degraded after 24 h treatment with AraC (Figure 21). Moreover, upon AraC treatment, CD157high cells retained some degree of GSK-3β phosphorylation at Ser9, which is known to increase the Mcl-1 stabilization [77], paralleled by the reduced cleavage of pro-apoptotic Bax, Caspase-3 and PARP-1 (Figure 21). These findings confirmed that CD157 signaling reduces the sensitivity of AML cells to AraC through the modulation of the intrinsic apoptotic pathway.

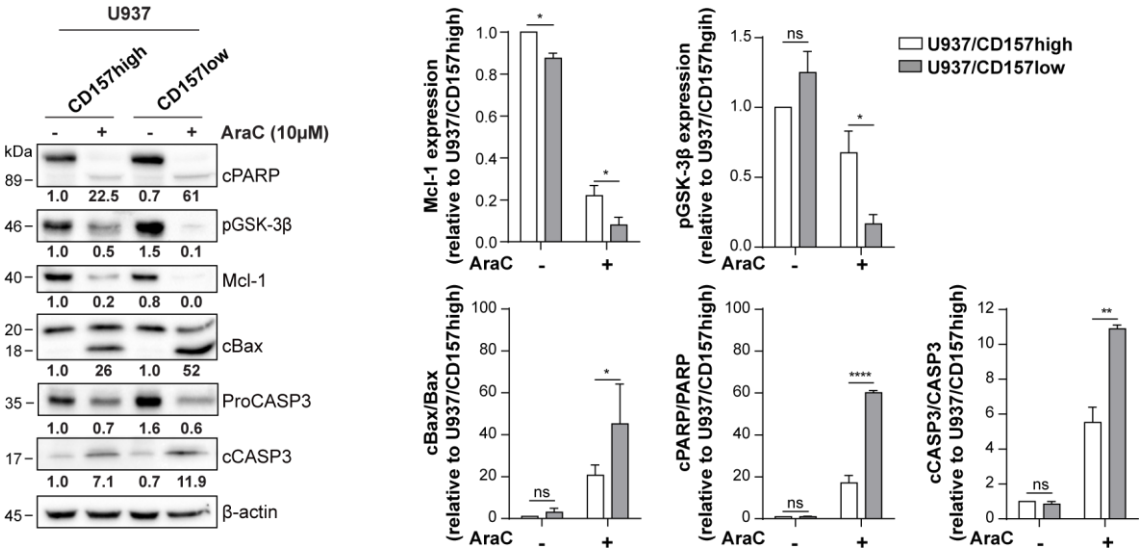


Figure 21. CD157 knockdown increases U937 cells AraC-induced cell death through the modulation of apoptosis. Right panel: representative Western blot analysis of U937/CD157high and CD157low cells treated with AraC for 24 h. β-actin was used as loading control. Numbers below blots indicate fold change in the expression of each protein relative to untreated CD157high cells, normalized to the corresponding β-actin and full length protein (PARP, Bax and Caspase-3). Left panels: histograms showing levels of indicated proteins determined by densitometry analysis expressed as the mean ± SEM of three experiments run in parallel. (*p <0.05, **p <0.01, ****p <0.0001, ns = not significant, two-way ANOVA test).

4.3.6 CD157 promotes survival of AML cells through Mcl-1 upregulation

Defects in apoptotic cell death can promote survival and impair responses of malignant cells to anti-cancer therapy [78]. We reasoned that CD157 could regulate the balance between survival versus death of AML cells by modulating the expression levels of selected Bcl-2 family proteins. To this aim, specific small molecule inhibitors, termed BH3-mimetics and targeting the apoptotic pathway were used. The Bcl-2 inhibitor ABT-199/venetoclax and the Mcl-1 inhibitor S63845 were directly compared in U937 cells engineered for CD157 expression. Consistent with previous observations [36], U937 cells were resistant to ABT-199 (Figure 22A), however, treatment of U937 cells with increasing concentration of the S63845 Mcl-1 inhibitor induced a dose-dependent cell death with high sensitivity. No significant correlation was found between sensitivity and CD157 expression (Figure 22B). Consequently, we focused on the Mcl-1 protein, which is commonly upregulated in AML cells, and is often a primary mode of resistance to treatment with the Bcl-2 inhibitor Venetoclax [79].

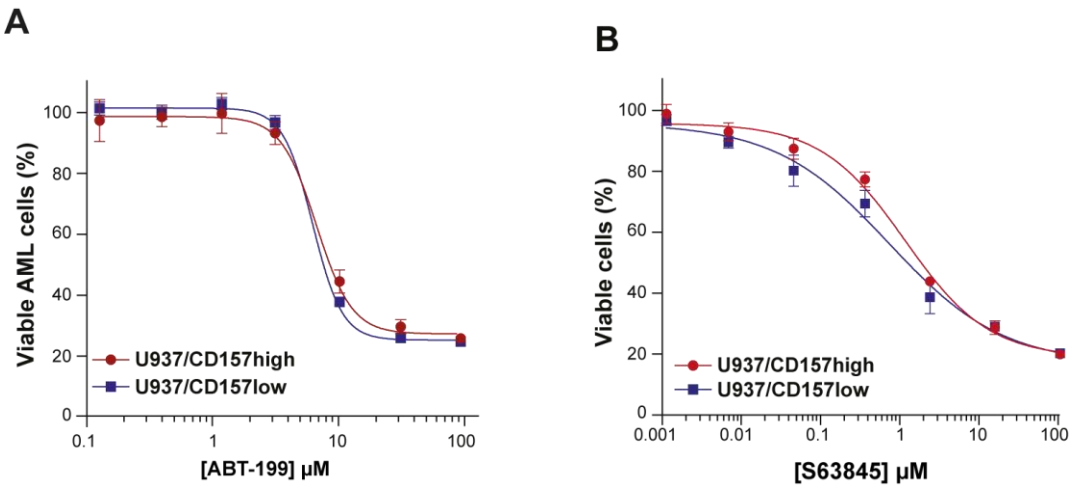


Figure 22. Effect of ABT-199 and S63845 on U937 viability. PrestoBlue assays showing the viability of CD157high (red line) versus CD157low (blue line) U937 cells after treatment with increasing concentrations of **A)** ABT-199 or **B)** S63845 for 24 h. Results are expressed as mean ± SEM of three independent experiments performed in six replicates.

To assess the relationship between CD157-mediated anti-apoptotic effect and Mcl-1, U937 cells were treated with 20 nM of the Mcl-1 inhibitor S63845 for 24, 48 or 72 h. Western blot showed that after 24 h, Mcl-1 protein levels increased in both U937/CD157high cells and U937/CD157low cells (Figure 23A), as S63845 inhibitor has been described to prolong Mcl-1 half-life [80]. However, the accumulation of Mcl-1 protein was greater in CD157high than in CD157low cells. After 48-72 h, Mcl-1 was progressively degraded, with more evident Caspase-3 and PARP-1 cleavage in CD157high cells, indicating induction of apoptosis. Consistently, after 72 h of exposure to S63845, the percentage of apoptotic cells was significantly higher in CD157high compared to CD157low U937 cells (Figure 23B).

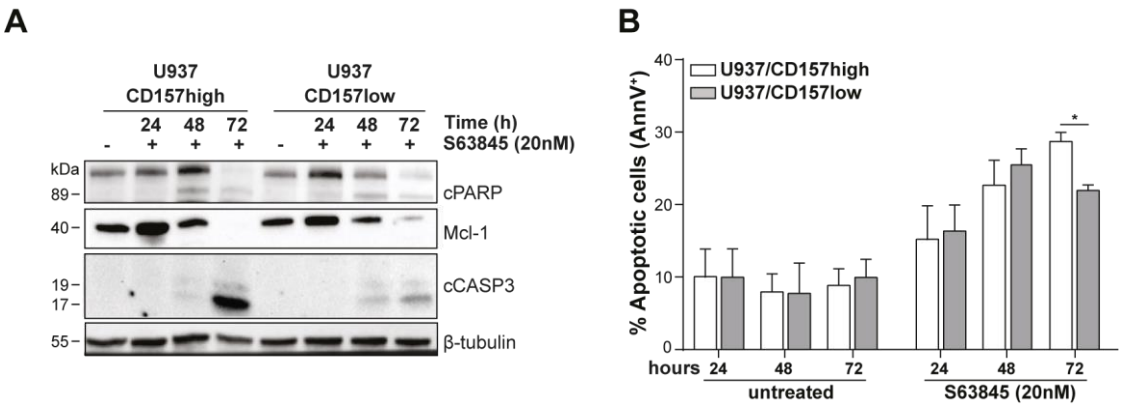


Figure 23. Effect of the S63845 Mcl-1 inhibitor on U937 cells engineered for CD157 expression. A) Western blot analysis of PARP, Mcl-1 and cleaved Caspase3 in U937/CD157high and CD157low cells treated with 20 nM S63845 for 24, 48 or 72 h. β-tubulin was used as loading control. **B)** AnnexinV/PI analysis of CD157high and CD157low U937 cells treated with S63845 up to 72 h. Histograms show the mean ± SEM of three independent experiments. (*p <0.05, two-way ANOVA test).

To confirm that Mcl-1 inhibitor displays on-target toxicity and that CD157high U937 cells depend on Mcl-1 for cell survival, we immunoprecipitated Mcl-1 protein using both U937 cells with high or low CD157. We found that Mcl-1 co-imunoprecipitated with Bim, whereas in CD157high cells Mcl-1 also sequestered Bak (Figure 24). This association was almost negligible in CD157low cells. Treatment with 200 nM S63845 displaced Bim from Mcl-1 and drastically destabilized Mcl-1/Bak association. Reciprocal immunoprecipitation using a Bim-specific antibody confirmed that in U937 cells Bim was mainly associated with Mcl-1, while it was only minimally associated with Bcl-2, regardless the expression of CD157.

These data support our hypothesis that CD157 facilitates the survival of leukemic blasts by interfering with the intrinsic apoptotic pathway through Mcl-1 upregulation, and suggests that sequestration of Bak by Mcl-1 might represent the primary CD157-mediated anti-apoptotic mechanism in AML cells.

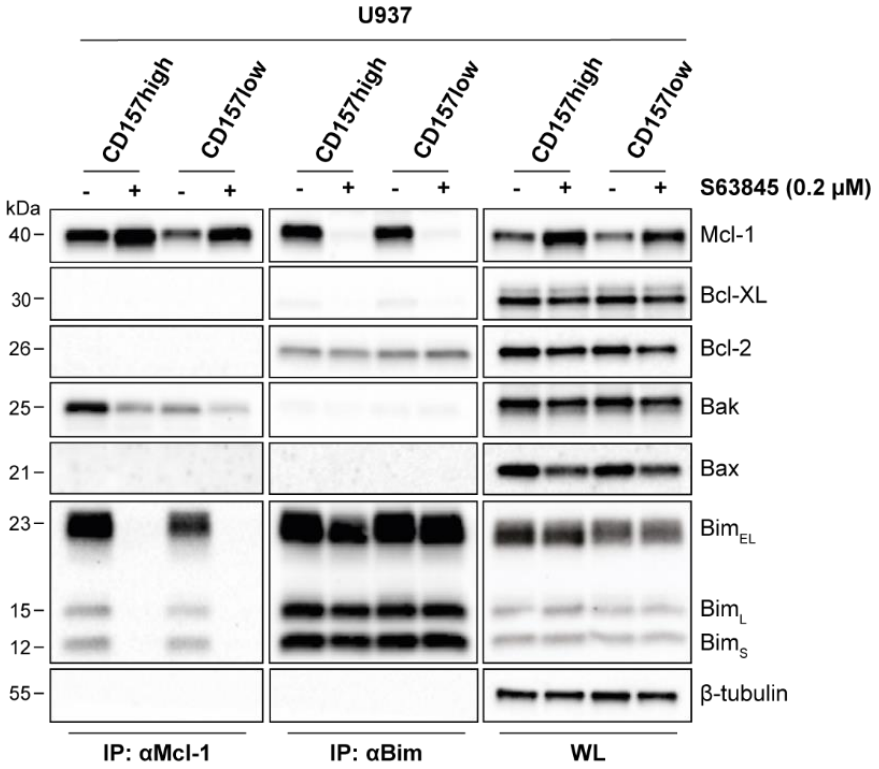


Figure 24. CD157-mediated protection of AML cells from apoptosis is partially dependent on Bak sequestration by Mcl-1. U937/CD157high and CD157low cells were treated with S63845 for 24 h, then cell lysates were immunoprecipitated with anti-Mcl-1 and anti-Bim antibodies, respectively. The interaction with the indicated proteins were probed by immunoblotting. β-tubulin was used as loading control. The whole cell lysates (WL) show the protein expression levels. One representative of three independent experiments is shown.

4.3.7 Mcl-1 inhibition improves therapeutic efficacy of AraC in CD157high AML cells

Overexpression of Mcl-1 has been implicated in resistance to both chemotherapy and BH3-mimetics targeting Bcl-2 and Bcl-XL [35]. The promising clinical activity of BH3-mimetics targeting Bcl-2 family proteins in AML treatment [81] prompted us to evaluate the effects of a combined therapy with the standard-of-care drug AraC and the Mcl-1 inhibitor S63845 in U937 cells with high and low CD157 expression. Cells were treated for 24 h with increasing concentrations of AraC and S63845 either as single agents or in combination, maintaining a constant ratio between the two drugs (S63845:AraC = 1:100). The results showed that combined treatment dramatically enhanced cell death of CD157high, but not of CD157low cells, at all doses considered, compared to each drug given alone (Figure 25A, B).

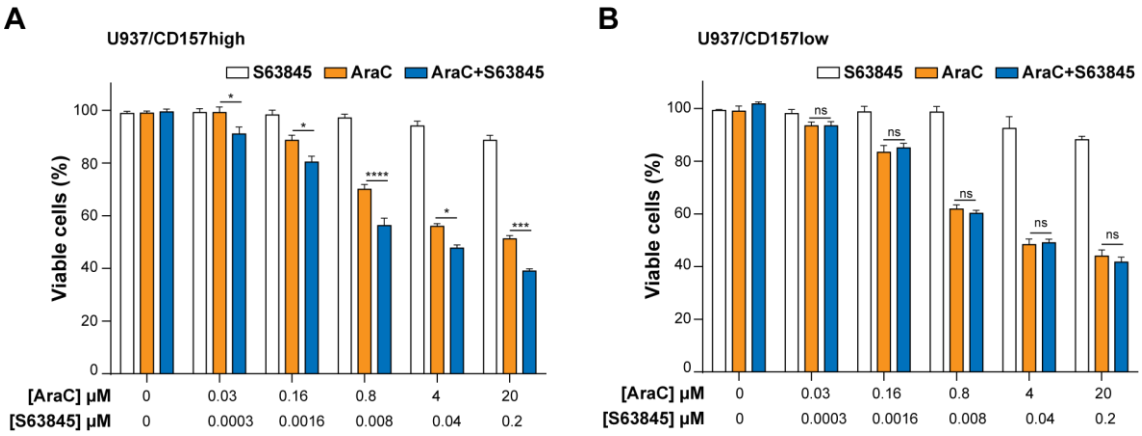


Figure 25. Effect of S63845 and AraC combination on U937 cell viability. A) U937/CD157high cells or **B)** U937/CD157low cells were treated for 24 h with increasing concentrations of S63845 or AraC as single drugs or in combination, at constant ratio S63845:AraC = 1:100. Cell viability was measured by PrestoBlue assays and was expressed as fold change of viable cells compared to untreated control cells. Histograms show the mean ± SEM of three independent experiments. (*p <0.05, ***p <0.01, ****p <0.001, ns = not significant, two-way ANOVA test).

To directly examine the signaling pathways activated by CD157 following the combined treatment, Western blot analysis of CD157high and low U937 cells, treated with 10 µM AraC and 0.2 µM S63845, was performed. As shown in Figure 26A, S63845 enhanced the activation of Caspase-3 induced by AraC, especially in U937/CD157high cells. Densitometric analysis confirmed that AraC-treated U937/CD157high cells had higher levels of Mcl-1, and lower levels of cleaved Caspase-3 (Figure 26B). This amply reflects the reduced sensitivity to AraC of CD157high cells.

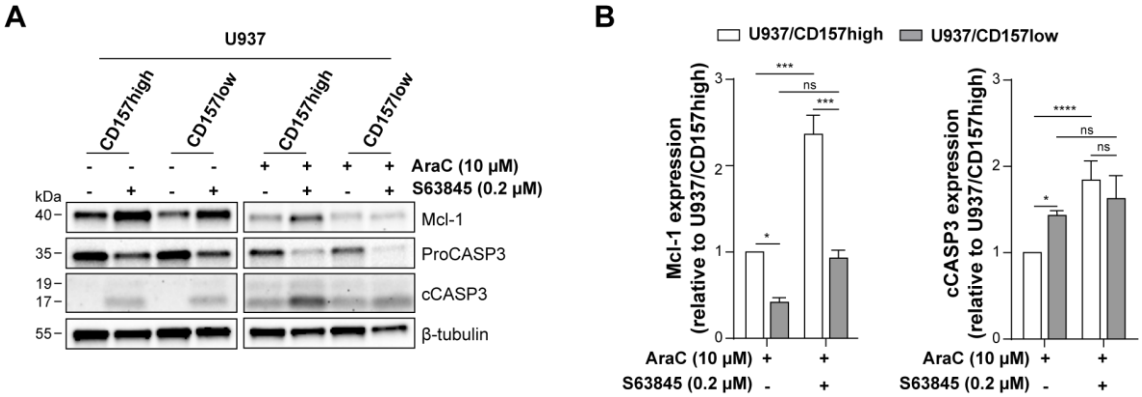


Figure 26. Mcl-1 inhibition increases AraC-induced apoptosis in CD157high U937 cells. A) Western blot analysis of CD157high or CD157low U937 cells treated for 24 h with S63845 or AraC as single agents or in combination. **B)** Densitometry analysis of Mcl-1 (left) and cleaved Caspase 3 (right) protein levels were compared in CD157high (white bars) and CD157low (gray bars) U937 cells. Results are means ± SEM of band densities of western blots from three independent experiments and are expressed as fold change over AraC-treated U937/CD157high cells. (*p <0.05, ***p <0.01, ****p <0.001, ns = not significant, two-way ANOVA test).

Indeed, the combination of AraC with S63845 had the best therapeutic index for CD157high cells and partially abolished the previously shown different sensitivity to AraC treatment between CD157high and CD157low cells (Figure 27).

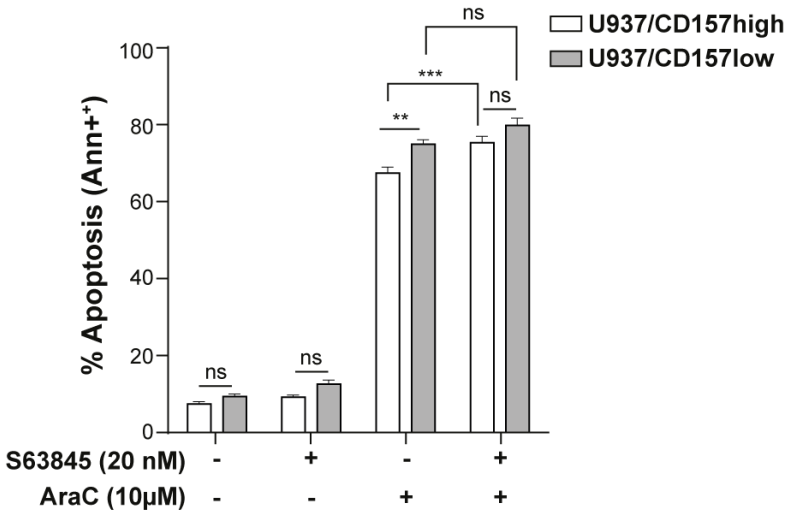


Figure 27. The combined treatment of AraC with S63845 restores the sensitivity to AraC in CD157high AML cells. AnnexinV/PI analysis of CD157high and CD157low U937 cells treated for 24 h with S63845 or AraC as single agents or in combination. Histograms show the percentage of apoptotic cells and are the mean ± SEM of eleven independent experiments. (**p <0.01, ***p <0.001, ns = not significant, two-way ANOVA test).

4.3 Role of CD157 in the interplay between AML cells and bone marrow niche

Over the last few years, several studies demonstrated a key role of the BM microenvironment in the pathogenesis of AML [82]. Cell–cell and cell–matrix interactions within the BM microenvironment significantly contribute to chemotherapy resistance and progression of AML. At the cellular level, this highly mutual interaction is granted by Cell Adhesion Molecules (CAMs) integrating differentiation, proliferation, and pro-survival signals from the surrounding microenvironment inside the cell and giving rise to the so-called cell adhesion-mediated drug resistance (CAM-DR) [83]. Given the AML dependence on the BM microenvironment, a better understanding of the interactions between the leukemic cells and the hematopoietic niche is needed, in order to develop therapies that co-target several signaling pathways simultaneously [82].

4.3.1 CD157 influences AML cell adhesion to ECM proteins

Functional characterization of CD157 in different physiological and pathological context, such as leukocyte trafficking and cancer metastasis, demonstrated that CD157 is involved in adhesion to and migration through selected ECM [63, 65]. In particular, CD157 controls leukocyte adhesion to extracellular matrix proteins, directional migration and diapedesis by establishing structural and functional interactions with specific members of the integrin family of adhesion receptors [61, 62]. Morone et al. demonstrated that CD157 binds with high affinity to selected ECM proteins, including fibronectin (FN), which proved to be its physiological ligand [67]. These observations lead us to investigate the role of CD157 in the interaction between leukemic cells and ECM proteins occurring within the leukemic niche.

To evaluate the involvement of CD157 in leukemic cells-ECM proteins interaction, we performed an adhesion assay by using U937 cells treated with a CD157 blocking antibody or anti-HLA Class I, anti-β1, α4 or α5 integrin mAbs, used as controls. Results showed that the adhesion of U937 cells to fibronectin was significantly inhibited by ligation of CD157 (Figure 28).

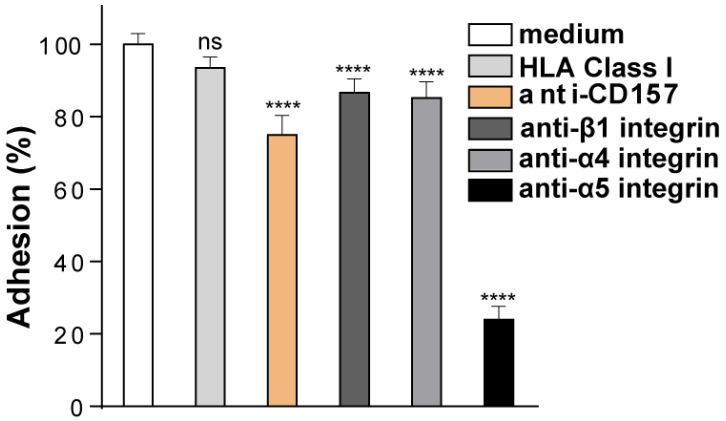


Figure 28. Effect of CD157 ligation on U937 cells adhesion to FN. Adhesion assay of U937 cells pretreated with anti-CD157 (SY11B5), anti-HLA Class I, anti-β1 integrin, anti-α4 integrin and anti-α5 integrin mAbs (all at 10μg/ml) for 20 minutes, then cells were seeded on FN-coated plates (10μg/ml) and incubated for 1 h at 37°C. The percentage of adhesion was calculated as the ratio between the calcein fluorescence intensity of cells after washing and the calcein fluorescence intensity of cells before washing. (****p <0.0001, ns = not significant, one-way ANOVA test).

To further investigate the contribution of CD157 in the interaction between leukemic cells and ECM proteins, we compared the adhesion efficiency of cells

engineered for CD157 expression to fibronectin. CD157 knockdown reduced the adhesion of THP1, U937 and OCI-AML3 cell lines to fibronectin (Figure 29A). Moreover, THP1/CD157low cells showed reduced adhesion to collagen type I (Coll I), and fibrinogen (FB), all binding CD157 with high affinity [67], but not to vitronectin (Vn), which is not a CD157 ligand (Figure 29B).

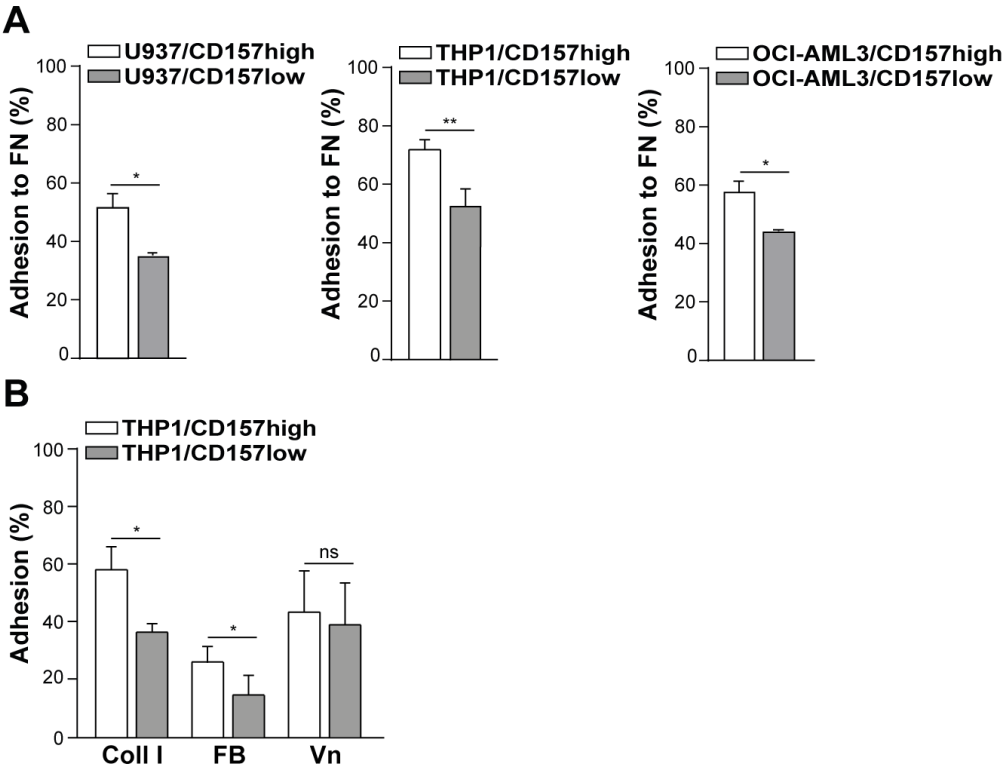


Figure 29. Adhesion of engineered AML cell lines to selected ECM proteins. A) CD157high and CD157low U937, THP1 and OCI-AML3 cell lines were seeded onto plates coated with FN (10 µg/ml) and incubated for 1 h at 37°C. **B)** Adhesion assay of THP1/CD157high and CD157low cells to Coll I, FB and Vn (all at 10 µg/ml) coated plates for 1h. Percentage of adhesion was calculated as the ratio between the fluorescence intensity of calcein-labelled cells after and before the washing step. Results are the mean ± SEM of three independent experiment. (*p <0.05, **p <0.01, ns = not significant, two-tailed unpaired t test).

The ability of CD157 to interact with selected ECM proteins led us to investigate if this interaction elicits a signaling cascade that involves the PI3K/Akt/mTOR pathway previously shown to be related to CD157-mediated pro-survival effect (See section 4.3.4). Hence, we investigated cell signaling events elicited by purified fibronectin, fibrinogen or collagen type I binding in CD157high or CD157low THP1 cells. Western blot analysis of cell extracts, obtained after treatment of cells with indicated ECM proteins for 10 min or 2 h, following overnight starvation, showed a higher phosphorylation of AKT, GSK-

3β and S6 ribosomal protein (especially after 10 min of exposure) and increased levels of Mcl-1 protein (after 2 h) in CD157high compared to CD157low cells (Figure 30).

Taken together, these results show that CD157 is involved in AML cells adhesion to ECM proteins and that the interaction of CD157 with selected ECM proteins supports the activation of pro-survival signals in AML cells.

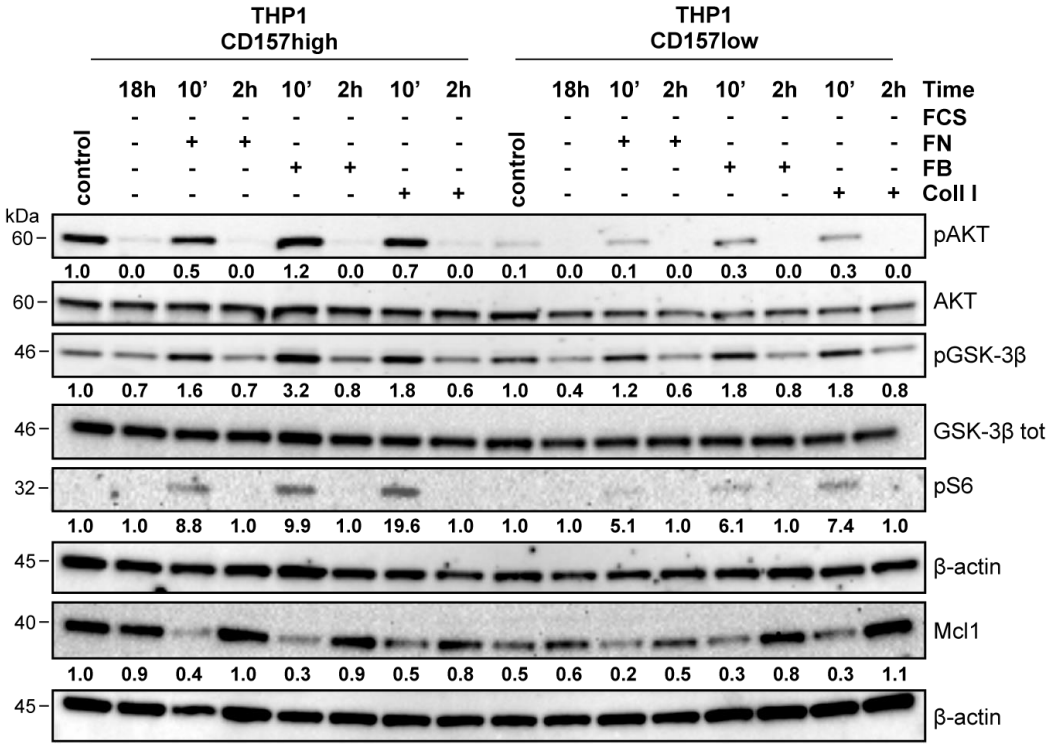


Figure 30. Intracellular pathway triggered by CD157 stimulation by ECM proteins. THP1/CD157high and THP1/CD157low cells were serum starved overnight and then stimulated with fibronectin (FN), fibrinogen (FB) or collagen type I (Coll I) (all at 20 μg/ml) for 10 min or 2 h. Numbers below blots indicate the fold change in the expression of each protein, compared to that of untreated control cells (CD157high), normalized to the corresponding β-actin and total proteins.

4.3.2 CD157 adhesion to fibronectin enhances the protective effect against AraC-induced cell death

The implication of CD157 expressed on AML cells in cell adhesion to FN and triggering of pro-survival signals mediated by CD157-FN interaction, fostered the hypothesis that CD157 could play a role in cell adhesion-mediated drug resistance (CAM-DR). To corroborate this hypothesis, CD157high and CD157low U937 cells were seeded onto FN-coated plates and then treated with AraC for 24 h. Results showed that CD157high cells were significantly more resistant to AraC treatment, as compared to CD157low cells (Figure 31A). To further confirm that CD157 takes part to FN-mediated protection of AML cells from AraC cytotoxicity, western blot analysis was performed in the same experimental conditions. Results showed that CD157high U937 cells grown on FN and treated with AraC, displayed lower levels of cleaved PARP-1, paralleled by higher phosphorylation of GSK-3β and enhanced Mcl-1 protein levels, as compared to either AraC alone, or to CD157low cells (Figure 31B).

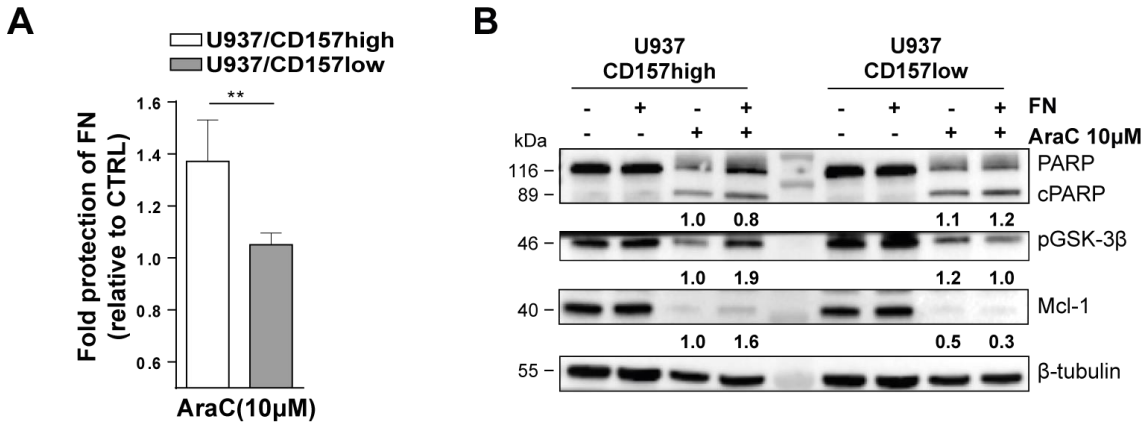


Figure 31. Effect of CD157 in fibronectin-mediated resistance to AraC in U937 cells. A) U937/CD157high and U937/CD157low cells were seeded onto plate coated with FN (10 μg/ml). After 2 h, 10 μM AraC was added and cell viability was determined after 24 h by PrestoBlue assays. Histograms represent the FN-mediated fold protection of cells treated with AraC compared to cells grown in the absence of FN. Results are the mean ± SEM of three independent experiments. (**p <0.01, two-tailed unpaired t test). **B)** Western blot analysis of CD157high or CD157low U937 cells seeded onto FN (10 μg/ml) and then treated with AraC for 24 h. β-tubulin was used as loading control. Numbers below blots indicate the fold change in the expression of each protein, compared to that of control cells (CD157high) treated with AraC, normalized to the corresponding β-tubulin.

4.4 CD157 takes part to the interaction between AML and bone marrow stromal cells

The interactions between leukemic cells and other cell types in the BM microenvironment are known to be crucial for the maintenance and progression of chemotherapy-resistant AML [84]. Among others, bone marrow stromal cells (BMSCs) play a critical role in the development, progression and relapse of AML, by leading leukemic cells toward a quiescent state, thus exerting a protective effect from drugs targeting rapidly dividing cells [85].

4.4.1 CD157 expression in BMSCs from AML patients

CD157 is expressed by multiple cellular components of the BM microenvironment [86]. Although it was originally described as bone marrow stromal marker [46], its expression and biological significance on BMSCs from AML patients has never been analysed. In order to assess the functional role of CD157 in the BMSCs-leukemic blasts crosstalk, we first analysed the expression of CD157 on BMSCs isolated from eight BM samples from primary AML by multiparametric flow cytometry analysis. BMSCs were identified by morphological parameters and by expression of CD90 BMSCs specific marker (Figure 32A). CD157 was found to be expressed in BMSCs of all patients analysed, although at variable levels (Figure 32B).

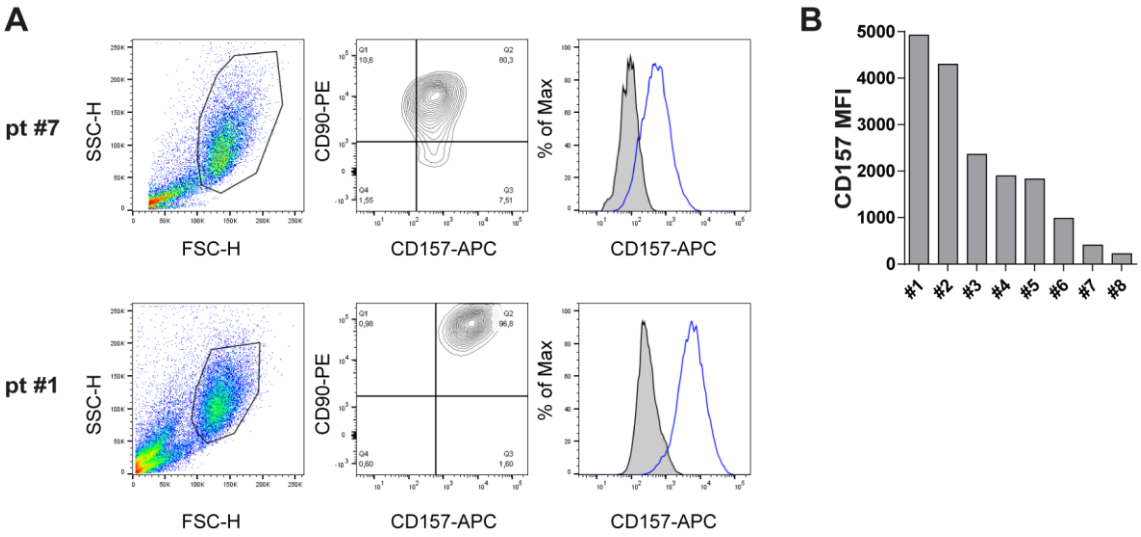


Figure 32. CD157 expression in primary BMSCs. A) Gating strategy for primary BMSCs analysis. BMSCs were selected based on their SSC and FSC parameters. CD90 expression was used to further characterize BMSCs population. FACS plots of CD157 expression in two representative samples of primary BMSCs are shown. Dot plots represent the morphological characteristics of BMSCs, while histograms show the expression levels of CD157 (blue empty peaks), compared to the isotype-matched control (grey full peaks). SSC, side scatter; FSC, forward scatter. **B)** The y-axis shows the mean fluorescence intensity (MFI) of CD157 expression on BMSCs in each patient (n = 8).

4.4.2 Generation of BMSCs engineered for the expression of CD157

Due to the limited number of primary BMSCs that can be obtained *in vitro*, to perform further functional experiments we used HS-5 immortalized normal human bone marrow stromal cells. Unlike primary BMSCs, the HS-5 cell line does not express CD157. Hence, to reproduce the phenotype of primary cells, we stably transfected HS-5 cells with a full-length cDNA sequence of CD157, or an empty vector, used as mock control (Figure 33).

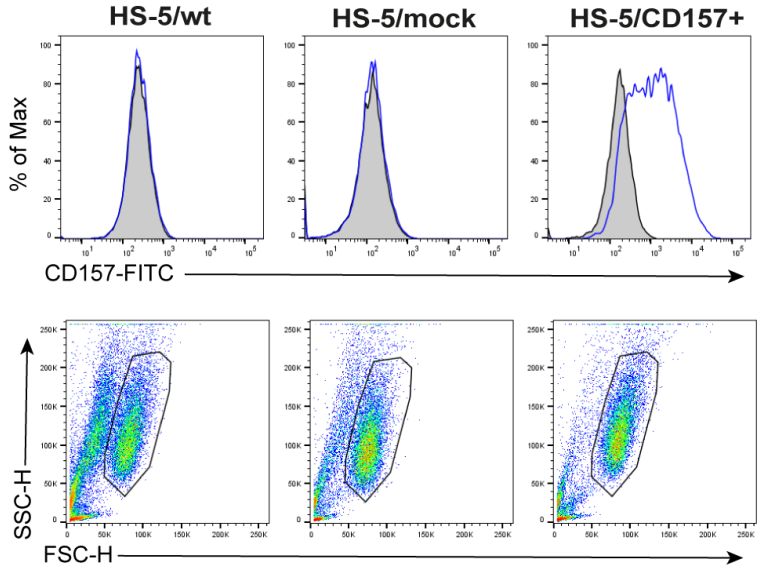


Figure 33. CD157 expression in HS-5 cells. Histograms show the expression of CD157 on the surface of wild type, mock and transfected HS-5 cells. Cells were stained with the anti-CD157 mAb (blue empty peaks) or with an isotype-matched mAb (grey full peaks). Dot plots represent the morphology of HS-5 cells.

4.4.3 Role of CD157 adhesion to BMSCs

The role of CD157 in the crosstalk between leukemic cells and BMSCs, was first evaluated in terms of adhesion efficiency of CD157high or CD157low U937 and THP1

cells to wild type HS-5 cells (HS-5/wt). Hence, a co-culture system with AML cells grown in contact with a monolayer of HS-5 cells was established. The results of the adhesion assay showed that the expression of CD157 by AML cells did not affect their adhesion efficiency to HS-5 cells (Figure 34).

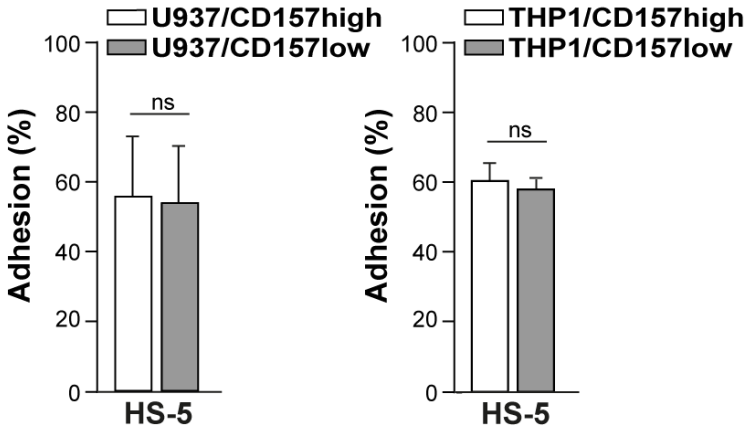


Figure 34. Adhesion of CD157high and CD157low AML cell lines to HS-5 cell line. CD157high and CD157low U937 (left panels) and THP1 (right panel) cells were seeded onto a monolayer of wild type HS-5 cells (CD157-negative) for 1h at 37°C. Percentage of adhesion was calculated as the ratio between the fluorescence intensity of calcein-labelled AML cells after and before the washing step. Results are the mean ± SEM of three independent experiment (ns = not significant, two-tailed unpaired t test).

Next, we focused on the contribution of CD157 expressed by HS-5 cells, by comparing the adhesion ability of U937 and THP1 cell lines (which constitutively expresses CD157) to CD157-positive or CD157-negative HS-5 stromal cells. When the AML cells were cultured in contact with HS-5/CD157+ cells, the number of adhered cells was significantly higher than those of AML cells co-cultured with HS-5/mock cells (Figure 35). Similar results were obtained with both THP1 and U937 AML cells. These data suggest that CD157 expressed by BMSCs takes part to the crosstalk between leukemic cells and BMSCs.

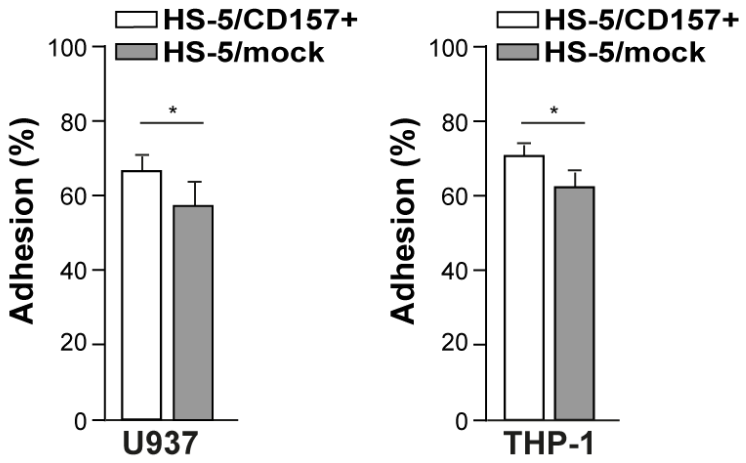


Figure 35. Adhesion of AML cells to CD157+ or CD157- HS-5 cell monolayers. U937 (left panels) and THP1 (right panel) cells were co-cultured onto a monolayer of HS-5/CD157+ and HS-5/mock cells for 1h at 37°C. Percentage of adhesion was calculated as the ratio between the fluorescence intensity of calcein-labelled AML cells after and before the washing step. Results are the mean ± SEM of three independent experiment (*$p < 0.05$, two-tailed unpaired t test).

4.4.4 CD157 contributes to the BMSCs-mediated drug resistance

Since the bone marrow microenvironment can protect AML blasts from chemotherapy, thus facilitating relapse [87], it was reasonable to predict that the co-culture of AML cells with HS-5 cells might increase CD157-mediated resistance to cytotoxic drugs. To substantiate this assumption, we co-cultured U937/CD157high and CD157low cells with BMSCs. After 48 h of treatment with 10 μM AraC, the viability of cells was assessed by AnnexinV/PI assay and FACS analysis. Results showed that the sensitivity of U937 cells to AraC decreased when cultured in contact with HS-5 cells (Figure 36). In line with previous results, the U937/CD157high cells resulted significantly less sensitive to AraC both in the presence or absence of HS-5 cells, compared to its CD157low counterpart. Noteworthy, HS-5 stromal cells exerted a protective effect toward AraC toxicity in U937/CD157high cells but not in U937/CD157low cells.

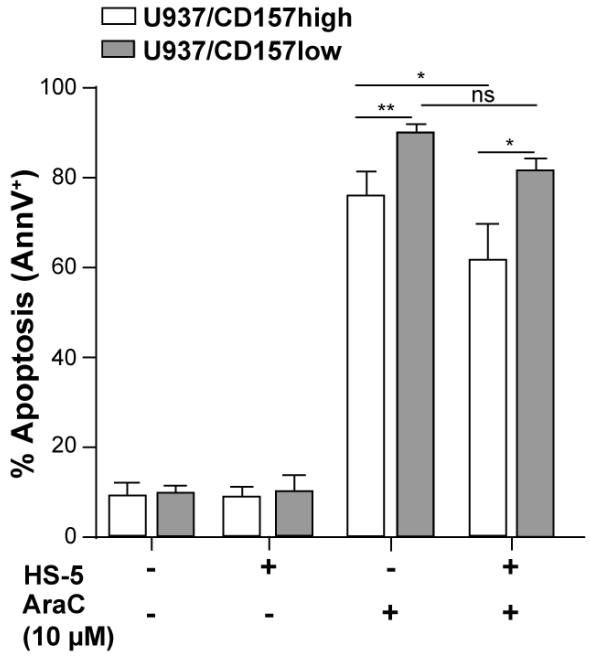


Figure 36. Effect of CD157 knockdown in HS-5-mediated chemoresistance in U937 cells. A) U937/CD157high and U937/CD157low cells cultured in suspension or co-cultured onto HS-5 cells were treated with 10 µM AraC for 48 h, then the percentage of apoptotic cells was determined by Annexin V/PI assay. Results are the mean ± SEM of three independent experiments (*p <0.05, **p <0.01, ns = not significant, two-tailed unpaired t test).

AraC is known to kill cancer cells by interfering with their DNA synthesis during the S phase of the cell cycle [88]. Therefore, to better define the contribution of CD157 in BMSCs-mediated protection of AML against AraC, we analyzed the cell cycle in U937 cells with high or low CD157. AML-HS-5 co-culture did not significantly affect cell cycle progression in U937/CD157low cells. Conversely, AraC-treated CD157high cells grown in contact with HS-5 cells predominantly accumulated in the G0/G1 phase, with a significant reduction in the number of cells in S phase (Figure 37). Taken together, these findings suggest that the interaction with BMSCs induces CD157high AML cells to accumulate in a quiescent low-cycling state, eventually leading to reduced cell sensitivity to AraC toxicity.

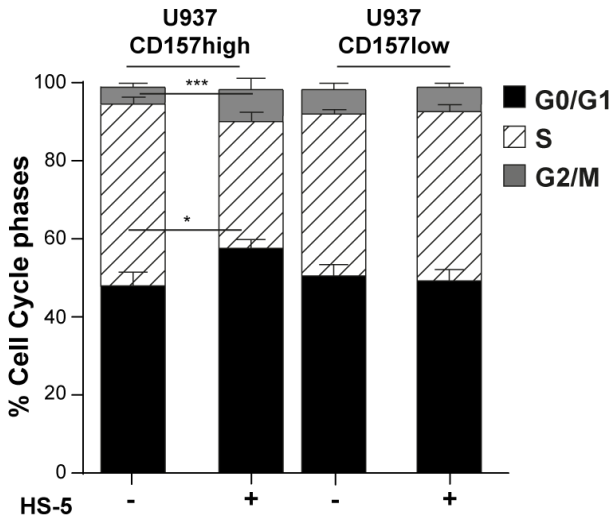


Figure 37. Impact of CD157 in cell cycle arrest mediated by BMSCs in AML cells. Co-culture assay showing the % of CD157high and CD157low U937 cells grown in contact with HS-5 cells in distinct phases of the cell cycle. The cell cycle of U937 cells was analysed by flow cytometry. Results are expressed as mean ± SEM of three independent experiments. (*p <0.05, ***p <0.001, two-tailed unpaired t test).

Since HS-5 cells do not express CD157, we performed similar experiments using engineered HS-5 CD157+ cells, in order to reproduce a more reliable scenario occurring within the bone marrow niche. Thus, we compared the cell cycle in U937 cells grown in co-culture with HS-5 cells expressing or not expressing CD157. Results confirmed that the co-culture of leukemic cells with the BMSCs increased the number of quiescent cells, compared to cells grown in the absence of BMSCs, regardless the expression of CD157 on BMSCs (Figure 38). However, the percentage of U937 cells accumulated in G0/G1 phase was remarkably higher when co-cultured with HS-5/CD157+ cells than with HS-5/CD157- cells (U937 alone = 52.3% ± 7.7 *versus* U937 co-cultured with HS-5/mock = 62.3% ± 5.4 *versus* U937 co-cultured with HS-5/CD157+ 67.2 ± 1.1).

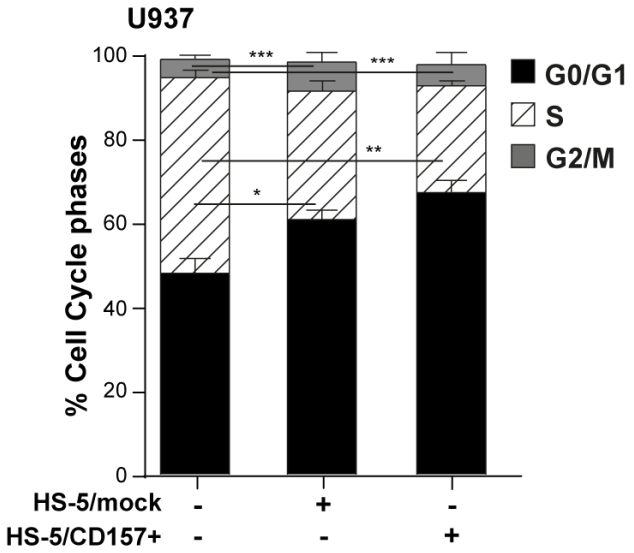


Figure 38. Impact of CD157 expressed by BMSCs on U937 cell cycle arrest. Co-culture assay showing the % of U937 cells in distinct phases of the cell cycle grown in contact with HS-5/mock or HS-5/CD157+ cells. After 48 h, the cell cycle of U937 cells was analysed by flow cytometry. Results are expressed as mean ± SEM of three independent experiments. (*p <0.05, **p <0.01, ***p <0.001, two-tailed unpaired t test).

Using the same co-culture conditions described above, the effect of CD157 expressed by BMSCs on the cytotoxic effect of AraC was subsequently evaluated. U937 cells were treated with 10µM AraC for 48 h. Consistently, BMSCs-mediated protection of AML cells from AraC cytotoxicity was independent of CD157 expression by BMSCs. Nevertheless, the protective effect against AraC mediated by HS-5/CD157+ cells was greater than that mediated by CD157-negative HS-5 stromal cells (Figure 39).

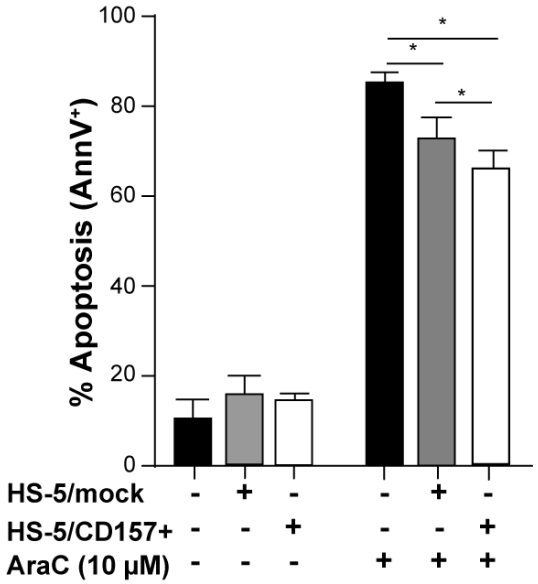


Figure 39. Effect of CD157 expression in HS-5 cells on protection of AML cells from chemotherapy. U937 cells were cultured in the presence or absence of HS-5/mock or HS-5/CD157+ cells and treated with 10 µM AraC for 48 h. The percentage of apoptotic cells was determined by Annexin V/PI assay. Results are the mean ± SEM of three independent experiments. (*p <0.01, two-tailed unpaired t test).

Overall, these findings suggest that CD157 expressed both by AML cells and BMSCs plays a role in the biology of AML dampening their sensitivity to AraC treatment.

5. Discussion

In this study we evaluated the expression and biological function of CD157 in AML by addressing our attention on its unique pattern of expression in both leukemic cells and BM stromal cells. In line with observation by other groups [58], the results from this study confirmed that CD157 is expressed in all patients with AML, regardless of subtype or genetic profile, both at diagnosis and relapse, although at variable epitope density. Overall, we unveiled an inverse correlation between the expression of CD157 and that of CD117 myeloid marker in AML blasts, hinting to an increased expression of CD157 by more differentiated myeloid blasts. This observations confirmed the original description of CD157 as myeloid differentiation marker [45, 89].

In addition, our functional studies revealed that CD157 favors survival and confers protection from stress or drug-induced apoptosis in primary AML blasts and AML cell lines. Two different experimental approaches support this notion: i) binding of CD157 by means of agonistic antibodies able to deliver intracellular signals, and ii) a genetic approach knocking down CD157 in AML cell line models. In detail, ligation of CD157 by a specific antibody supported survival and reduced AraC-induced apoptosis in primary AML cells *ex vivo*. Indeed, in agreement with previous studies clearly delineating the ability of CD157 to act as a signaling molecule [62, 63, 90], our data showed that CD157 exerts its pro-survival effect in AML by transducing signals from external cues. Results from *in vitro* experiments corroborated this assumption showing that in primary AML cells, CD157 stimulation by antibody binding activated the PI3K/Akt/mTOR and MAPK/ERK pathways, while inactivating GSK3-β kinase. This resulted in an extended *ex vivo* blast survival and reduced sensitivity to AraC treatment. In addition, using a genetic approach, a stronger phosphorylation of Akt and its downstream effectors was found in CD157high compared to CD157low AML cell lines, exposed to FCS (as source of ECM proteins) or to soluble purified ECM proteins able to bind CD157 with high affinity [67]. PI3K/Akt/mTOR and MAPK/ERK intracellular signaling pathways, as well as GSK3-β, are key regulators of cell growth, survival and apoptosis, and their activation has been implicated in both the pathogenesis and progression of AML [41]. In leukemic cells, aberrant activation of these intracellular signals converge towards apoptotic escape, favoring tumor growth and eventually leading to resistance to chemotherapy [91, 92]. Upon its activation, Akt can phosphorylate numerous downstream functional targets, including GSK-3β, thereby affecting cell metabolism, survival and proliferation [93, 94].

GSK3-β is a multifunctional serine/threonine kinase involved in a number of cellular processes, including differentiation, signal transduction, cell cycle regulation and proliferation [92]. The results of this study unveiled that the intracellular signals elicited by CD157 ligation determined inactivation of GSK-3β through phosphorylation at Ser9, which leads to increased accumulation of Mcl-1 and Bcl-XL anti-apoptotic proteins. In contrast, these signals decreased Bax pro-apoptotic protein and reduced Caspase-3 activation, thus dampening the apoptotic response in primary AML cells [77]. In keeping with these findings, knockdown of CD157 increased apoptosis in U937 and THP1 AML cell lines exposed to nutrient deprivation conditions, reduced Mcl-1 protein levels, and resulted in increased sensitivity to AraC toxicity in U937 cells. In U937 cells Mcl-1 proved to be primarily bound to the BH3-only pro-apoptotic protein Bim, while its binding to Bcl-2 was barely detectable. Of note, in CD157high U937 cells the pro-apoptotic effector protein Bak was sequestered by Mcl-1 which precludes the activation of Bax/Bak-dependent mitochondrial apoptosis [95]. Consistently, treatment with the Mcl-1 inhibitor S63845, which binds with high affinity to the BH3-binding groove of Mcl-1 [43], displaced Bim and Bak from Mcl-1, allowing the activation of the Bax/Bak-mediated apoptosis [43] (Figure 40). Used in combination with AraC, very low doses of S63845 were sufficient to significantly increase the therapeutic efficacy of AraC in CD157high but not in CD157low AML cells.

Taken together, these findings highlighted that CD157-mediated signaling cascade upregulates Mcl-1 anti-apoptotic protein, thus helping leukemia cells to escape apoptosis and raising their sensitivity threshold to AraC treatment, at least *in vitro*. However, the small number of samples analysed in *ex vivo* experiments does not allow to drawn definitive conclusions.

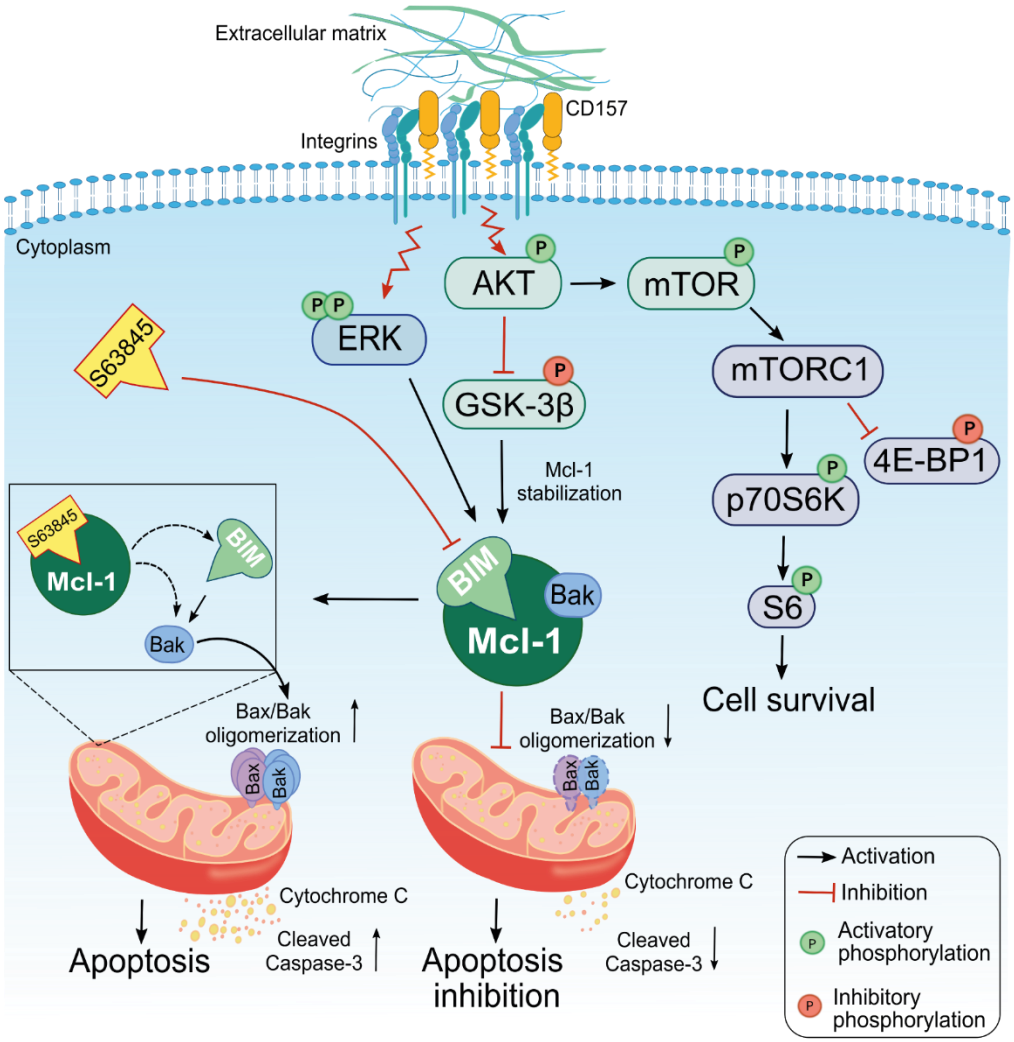


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Figure 40. Schematic representation of CD157-mediated survival in AML cells. CD157 binding to fibronectin in AML cells promotes structural and functional interaction of CD157 with specific integrins. This activates intracellular signals leading to phosphorylation of AKT, mTOR and its downstream substrates p70S6K, pS6 ribosomal protein, 4E-BP1, and ERK. In turn, phosphorylated AKT inactivates its major target GSK-3β, which loses its pro-apoptotic function and favors the stabilization of Mcl-1. The increased expression of Mcl-1 strengthens its ability to sequester Bim and Bak, thus precluding the Bak/Bax oligomerization at the mitochondrial outer membrane, which is required for cytochrome c release and is considered a key step in apoptosis. S63845 binds to the BH3-binding groove of Mcl-1 releasing Bim and Bak and enabling the activation of the Bax/Bak-dependent apoptotic pathway. This results in outer mitochondrial membrane permeabilization and release of cytochrome c into the cytosol leading to activation of the caspase cascade [96].

Recent clinical applications of the Bcl-2 inhibitor venetoclax (ABT-199) demonstrated that venetoclax-based regimens are very effective in many patients. However, clinical data highlighted that a non-negligible number of patients do not

respond or do eventually relapse after achieving remission, indicating that there are different subgroups in AML with reliance on different anti-apoptotic proteins for survival [81]. In particular, the upregulation of Mcl-1 is considered the main way through which AML are or become resistant to venetoclax-based therapy [97]. Mcl-1 may also play a key role in determining the effect of venetoclax in specific AML morphologic subtypes [39]. It has been shown that AML with monocytic differentiation characteristics are resistant to venetoclax-based therapies. This is likely attributable to the high expression of Mcl-1 in monocytic blasts, whose survival preferentially rely on Mcl-1 [98]. Noteworthy, CD157 expression is significantly higher in differentiated monocytic blasts (i.e., in FAB M4 and M5 subtypes) than in all other less differentiated AML subtypes [58]. It is tempting to speculate that high levels of CD157 expression could be a useful tool for the prospective identification of patients with a disease phenotype who are unlikely to respond to Bcl-2 inhibitors, but most likely to benefit from Mcl-1-specific inhibitors. This hypothesis requires further study both *ex vivo* and *in vivo* to verify whether CD157 is a prognostic or predictive factor to Mcl-1 and Bcl-2 inhibitors clinical response that may contribute to stratify patient's risk optimizing the treatment approach. However, it is reasonable to assume that in addition to CD157 expression, other molecular characteristics, such as genetic mutations, must be considered to better define homogeneous subgroups of patients. So far, no correlations have emerged between genetic profile and CD157 expression levels. However, the number of cases analysed in our study or reported by others is very limited [58], therefore it is necessary to extend the analysis before any conclusions can be drawn.

Compelling evidence supports the hypothesis that the interactions between leukemia cells and extracellular matrix proteins or BM stromal cells result in a protective environment favoring tumor development and facilitating resistance to cytotoxic treatments [99]. CD157 expression in both leukemic blasts and bone marrow stromal cells led us to investigate the potential involvement of CD157 in the cross-talk between tumor and BM microenvironment in AML. Experimental data showed that the interaction of CD157 with selected extracellular matrix proteins (including fibronectin, fibrinogen and collagen type I), known to bound CD157 with high affinity and abundantly produced by stromal cell *in vivo*, produces effects similar to those we have previously observed *in vitro* using an agonistic anti-CD157 antibody as a surrogate of the physiological ligand. First, we observed that both genetic loss and CD157 binding by agonistic antibodies reduced the adhesion of leukemic cells to fibronectin and other selected ECM proteins, suggesting

a role of CD157 in blast homing and anchoring within the BM niche. Moreover, adhesion to FN proved to be more efficient in CD157high than in CD157low AML cells and this was accompanied by reduced sensitivity of CD157high AML cells to AraC cytotoxicity. This is consistent with numerous evidence indicating that the interaction with FN protects AML cells from AraC-induced apoptosis [100]. Consequently, the greater the interaction with FN, the greater the protection from AraC toxicity is expected. However, the molecular mechanism through which CD157 exerts its protective effect against chemotherapy in BM environment is not fully clarified. It is likely that functional and structural interactions between CD157 and integrins implemented *in vivo* by ECM proteins [62], trigger an aberrant activation of the PI3K/Akt signaling pathway promoting cell survival while reducing drug sensitivity.

BMSCs have an established protective effect against drug-induced apoptosis and control leukemia cells maintenance and survival [101]. We showed that CD157 is expressed by BMSCs from all AML patients analysed, strengthening our hypothesis of its involvement in tumor-stroma interactions. The *in vitro* co-culture system adopted in this study, allowed us to unravel the impact of CD157 on both leukemia cells and stromal cells. Indeed, although CD157 expressed by AML cells did not influence cell-cell adhesion, it significantly increased the stroma-mediated protective effect against AraC toxicity. At the molecular level, this protective effect is supported by the accumulation of leukemic cells in the G0/G1 phase (indicating cell cycle arrest), hinting to a role of CD157 in the accumulation of quiescent leukemia cells within the BM microenvironment. The induction of cell quiescence is a well-known mechanism through which BMSCs protect leukemic cells from chemotherapeutic drugs - such as AraC - that kill actively proliferating cells [99].

When both leukemic and HS-5 stromal cells expressed CD157 (a scenario recapitulating what happens *in vivo*) the induction of cell quiescence was exacerbated, as demonstrated by a greater number of cells in the G0/G1 phase, with a concomitant reduction of the number of AML cells in the S phase, compared to CD157-negative HS-5 stromal cells. Taken together, these findings suggest that CD157 expressed both by tumor cells and by the surrounding microenvironment takes part to the dialogue between AML cells and tumor microenvironment thus contributing to CAM-DR. However, these observations require further study to explore its translational relevance by using primary samples from patients with AML.

In summary, the peculiar expression of CD157 both in leukemic blasts and in bone marrow stromal cells led us to hypothesize that it could have a role on both sides. This was indeed the case, since not only CD157 modulates the vitality and sensitivity to chemotherapy of blasts, but it also has a central role in the interaction between blasts and neighboring stromal cells in the bone marrow. These findings suggest that CD157 can be a promising candidate to develop therapies that simultaneously target leukemia cells and BM niche. Anyhow, a note of caution in required, because CD157 expression is not restricted to leukemic cells, but also extends to normal mature and immature myeloid cells and vascular endothelial cells, possibly causing undesirable side-effects, hinting that optimization of the clinical use of CD157-specific therapies could be challenging. The results of Phase I clinical trial for relapsed/refractory AML (NCT02353143) with MEN1112/OBT357, a first humanized de-fucosylated IgG1 mAb targeting CD157 [58], has provided initial evidence of unpredictable liver toxicity and meaningful target engagement, and was ultimately terminated. It is likely that the biological functions of CD157 in AML need to be deeply studied before exploiting this molecule in clinical practice. However, development of targeted antibody-based immunotherapeutic agents is an area of growing interest in AML. Regulatory history and challenges of Gemtuzumab ozogamicin (GO, the only one antibody approved for therapy in AML) are an important reminder of the complexity of drugs development [102]. As we learned from the history of GO, it will be important to fully evaluate not only the use of the drug but also how it is dosed, at what time it is given during treatment, at what frequency, and with what other combinations in order to ensure the maximum patient benefit to improve AML cure rates.

6. References

1. *Surveillance, Epidemiology, and End Results (SEER) Program*. 2021, National Cancer Institute: Bethesda, MD,USA.
2. Short, N.J., M.E. Rytting, and J.E. Cortes, *Acute myeloid leukaemia.* Lancet, 2018. **392**(10147): p. 593-606.
3. Song, X., et al., *Incidence, Survival, and Risk Factors for Adults with Acute Myeloid Leukemia Not Otherwise Specified and Acute Myeloid Leukemia with Recurrent Genetic Abnormalities: Analysis of the Surveillance, Epidemiology, and End Results (SEER) Database, 2001-2013.* Acta Haematol, 2018. **139**(2): p. 115-127.
4. Vago, L. and I. Gojo, *Immune escape and immunotherapy of acute myeloid leukemia.* J Clin Invest, 2020. **130**(4): p. 1552-1564.
5. Lane, S.W. and D.G. Gilliland, *Leukemia stem cells.* Semin Cancer Biol, 2010. **20**(2): p. 71-6.
6. Pollyea, D.A. and C.T. Jordan, *Therapeutic targeting of acute myeloid leukemia stem cells.* Blood, 2017. **129**(12): p. 1627-1635.
7. Davis, J.R., D.J. Benjamin, and B.A. Jonas, *New and emerging therapies for acute myeloid leukaemia.* J Investig Med, 2018. **66**(8): p. 1088-1095.
8. Sekeres, M.A., et al., *American Society of Hematology 2020 guidelines for treating newly diagnosed acute myeloid leukemia in older adults.* Blood Adv, 2020. **4**(15): p. 3528-3549.
9. Gurnari, C., et al., *From Bench to Bedside and Beyond: Therapeutic Scenario in Acute Myeloid Leukemia.* Cancers (Basel), 2020. **12**(2).
10. Saultz, J.N. and R. Garzon, *Acute Myeloid Leukemia: A Concise Review.* J Clin Med, 2016. **5**(3).
11. Dohner, H., et al., *Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel.* Blood, 2017. **129**(4): p. 424-447.
12. Bene, M.C., et al., *Immunophenotyping of acute leukemia and lymphoproliferative disorders: a consensus proposal of the European LeukemiaNet Work Package 10.* Leukemia, 2011. **25**(4): p. 567-74.
13. De Kouchkovsky, I. and M. Abdul-Hay, *'Acute myeloid leukemia: a comprehensive review and 2016 update'.* Blood Cancer J, 2016. **6**(7): p. e441.
14. Bennett, J.M., et al., *Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group.* Br J Haematol, 1976. **33**(4): p. 451-8.
15. Hwang, S.M., *Classification of acute myeloid leukemia.* Blood Res, 2020. **55**(S1): p. S1-S4.
16. Vosberg, S. and P.A. Greif, *Clonal evolution of acute myeloid leukemia from diagnosis to relapse.* Genes Chromosomes Cancer, 2019. **58**(12): p. 839-849.
17. Jan, M., et al., *Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia.* Sci Transl Med, 2012. **4**(149): p. 149ra118.
18. Shallis, R.M., et al., *Epidemiology of acute myeloid leukemia: Recent progress and enduring challenges.* Blood Rev, 2019. **36**: p. 70-87.
19. Kuykendall, A., et al., *Acute Myeloid Leukemia: The Good, the Bad, and the Ugly.* Am Soc Clin Oncol Educ Book, 2018. **38**: p. 555-573.
20. Metzeler, K.H., et al., *Spectrum and prognostic relevance of driver gene mutations in acute myeloid leukemia.* Blood, 2016. **128**(5): p. 686-98.

21. El Achi, H. and R. Kanagal-Shamanna, *Biomarkers in Acute Myeloid Leukemia: Leveraging Next Generation Sequencing Data for Optimal Therapeutic Strategies.* Front Oncol, 2021. **11**: p. 748250.
22. Cancer Genome Atlas Research, N., et al., *Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia.* N Engl J Med, 2013. **368**(22): p. 2059-74.
23. Papaemmanuil, E., et al., *Genomic Classification and Prognosis in Acute Myeloid Leukemia.* N Engl J Med, 2016. **374**(23): p. 2209-2221.
24. Ahmadmehrabi, K., et al., *Targeted Therapies for the Evolving Molecular Landscape of Acute Myeloid Leukemia.* Cancers (Basel), 2021. **13**(18).
25. Mrozek, K., et al., *Prognostic significance of the European LeukemiaNet standardized system for reporting cytogenetic and molecular alterations in adults with acute myeloid leukemia.* J Clin Oncol, 2012. **30**(36): p. 4515-23.
26. Bose, P., P. Vachhani, and J.E. Cortes, *Treatment of Relapsed/Refractory Acute Myeloid Leukemia.* Curr Treat Options Oncol, 2017. **18**(3): p. 17.
27. DiNardo, C.D. and A.E. Perl, *Advances in patient care through increasingly individualized therapy.* Nat Rev Clin Oncol, 2019. **16**(2): p. 73-74.
28. Tang, K., A.C. Schuh, and K.W. Yee, *3+7 Combined Chemotherapy for Acute Myeloid Leukemia: Is It Time to Say Goodbye?* Curr Oncol Rep, 2021. **23**(10): p. 120.
29. Gurnari, C., S. Pagliuca, and V. Visconte, *Deciphering the Therapeutic Resistance in Acute Myeloid Leukemia.* Int J Mol Sci, 2020. **21**(22).
30. Liu, H., *Emerging agents and regimens for AML.* J Hematol Oncol, 2021. **14**(1): p. 49.
31. Stone, R.M., et al., *Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation.* N Engl J Med, 2017. **377**(5): p. 454-464.
32. Lambert, J., et al., *Gemtuzumab ozogamicin for de novo acute myeloid leukemia: final efficacy and safety updates from the open-label, phase III ALFA-0701 trial.* Haematologica, 2019. **104**(1): p. 113-119.
33. DiNardo, C.D., et al., *Durable Remissions with Ivosidenib in IDH1-Mutated Relapsed or Refractory AML.* N Engl J Med, 2018. **378**(25): p. 2386-2398.
34. Stein, E.M., et al., *Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia.* Blood, 2017. **130**(6): p. 722-731.
35. Konopleva, M., et al., *Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia.* Cancer Cell, 2006. **10**(5): p. 375-88.
36. Greaves, G., et al., *BH3-only proteins are dispensable for apoptosis induced by pharmacological inhibition of both MCL-1 and BCL-XL.* Cell Death Differ, 2019. **26**(6): p. 1037-1047.
37. Bose, P., V. Gandhi, and M. Konopleva, *Pathways and mechanisms of venetoclax resistance.* Leuk Lymphoma, 2017. **58**(9): p. 1-17.
38. Koss, B., et al., *Defining specificity and on-target activity of BH3-mimetics using engineered B-ALL cell lines.* Oncotarget, 2016. **7**(10): p. 11500-11.
39. Saxena, K., et al., *Harnessing Apoptosis in AML.* Clin Lymphoma Myeloma Leuk, 2021.
40. Pollyea, D.A., et al., *Venetoclax for AML: changing the treatment paradigm.* Blood Adv, 2019. **3**(24): p. 4326-4335.
41. Testa, U. and R. Riccioni, *Deregulation of apoptosis in acute myeloid leukemia.* Haematologica, 2007. **92**(1): p. 81-94.

42. Kotschy, A., et al., *The MCL1 inhibitor S63845 is tolerable and effective in diverse cancer models.* Nature, 2016. **538**(7626): p. 477-482.
43. Ewald, L., et al., *Side-by-side comparison of BH3-mimetics identifies MCL-1 as a key therapeutic target in AML.* Cell Death Dis, 2019. **10**(12): p. 917.
44. D'Aguanno, S. and D. Del Bufalo, *Inhibition of Anti-Apoptotic Bcl-2 Proteins in Preclinical and Clinical Studies: Current Overview in Cancer.* Cells, 2020. **9**(5).
45. Todd, R.F., 3rd, J.A. Roach, and M.A. Arnaout, *The modulated expression of Mo5, a human myelomonocytic plasma membrane antigen.* Blood, 1985. **65**(4): p. 964-73.
46. Kaisho, T., et al., *BST-1, a surface molecule of bone marrow stromal cell lines that facilitates pre-B-cell growth.* Proc Natl Acad Sci U S A, 1994. **91**(12): p. 5325-9.
47. Ishihara, K. and T. Hirano, *BST-1/CD157 regulates the humoral immune responses in vivo.* Chem Immunol, 2000. **75**: p. 235-55.
48. Quarona, V., et al., *CD38 and CD157: a long journey from activation markers to multifunctional molecules.* Cytometry B Clin Cytom, 2013. **84**(4): p. 207-17.
49. Hirata, Y., et al., *ADP ribosyl cyclase activity of a novel bone marrow stromal cell surface molecule, BST-1.* FEBS Lett, 1994. **356**(2-3): p. 244-8.
50. Yamamoto-Katayama, S., et al., *Site-directed removal of N-glycosylation sites in BST-1/CD157: effects on molecular and functional heterogeneity.* Biochem J, 2001. **357**(Pt 2): p. 385-92.
51. Lee, B.O., et al., *Elevated levels of the soluble form of bone marrow stromal cell antigen 1 in the sera of patients with severe rheumatoid arthritis.* Arthritis Rheum, 1996. **39**(4): p. 629-37.
52. Augeri, S., et al., *Soluble CD157 in pleural effusions: a complementary tool for the diagnosis of malignant mesothelioma.* Oncotarget, 2018. **9**(32): p. 22785-22801.
53. Chauhan, S., et al., *Surface Glycoproteins of Exosomes Shed by Myeloid-Derived Suppressor Cells Contribute to Function.* J Proteome Res, 2017. **16**(1): p. 238-246.
54. Ortolan, E., et al., *CD157: From immunoregulatory protein to potential therapeutic target.* Immunol Lett, 2019. **205**: p. 59-64.
55. Ortolan, E., et al., *Functional role and prognostic significance of CD157 in ovarian carcinoma.* J Natl Cancer Inst, 2010. **102**(15): p. 1160-77.
56. Ortolan, E., et al., *CD157 enhances malignant pleural mesothelioma aggressiveness and predicts poor clinical outcome.* Oncotarget, 2014. **5**(15): p. 6191-205.
57. Mirkowska, P., et al., *Leukemia surfaceome analysis reveals new disease-associated features.* Blood, 2013. **121**(25): p. e149-59.
58. Krupka, C., et al., *Targeting CD157 in AML using a novel, Fc-engineered antibody construct.* Oncotarget, 2017. **8**(22): p. 35707-35717.
59. Yakymiv, Y., et al., *CD157: From Myeloid Cell Differentiation Marker to Therapeutic Target in Acute Myeloid Leukemia.* Cells, 2019. **8**(12).
60. Huntly, B.J. and D.G. Gilliland, *Leukaemia stem cells and the evolution of cancer-stem-cell research.* Nat Rev Cancer, 2005. **5**(4): p. 311-21.
61. Lavagno, L., et al., *CD157 is part of a supramolecular complex with CD11b/CD18 on the human neutrophil cell surface.* J Biol Regul Homeost Agents, 2007. **21**(1-2): p. 5-11.

62. Lo Buono, N., et al., *The CD157-integrin partnership controls transendothelial migration and adhesion of human monocytes.* J Biol Chem, 2011. **286**(21): p. 18681-91.
63. Funaro, A., et al., *CD157 is an important mediator of neutrophil adhesion and migration.* Blood, 2004. **104**(13): p. 4269-78.
64. Funaro, A., et al., *Ectoenzymes and innate immunity: the role of human CD157 in leukocyte trafficking.* Front Biosci (Landmark Ed), 2009. **14**: p. 929-43.
65. Ortolan, E., et al., *CD157 plays a pivotal role in neutrophil transendothelial migration.* Blood, 2006. **108**(13): p. 4214-22.
66. Sutherland, D.R., et al., *Use of CD157 in FLAER-based assays for high-sensitivity PNH granulocyte and PNH monocyte detection.* Cytometry B Clin Cytom, 2014. **86**(1): p. 44-55.
67. Morone, S., et al., *Binding of CD157 protein to fibronectin regulates cell adhesion and spreading.* J Biol Chem, 2014. **289**(22): p. 15588-601.
68. Morone, S., et al., *Overexpression of CD157 contributes to epithelial ovarian cancer progression by promoting mesenchymal differentiation.* PLoS One, 2012. **7**(8): p. e43649.
69. van de Loosdrecht, A.A., et al., *Standardization of flow cytometry in myelodysplastic syndromes: report from the first European LeukemiaNet working conference on flow cytometry in myelodysplastic syndromes.* Haematologica, 2009. **94**(8): p. 1124-34.
70. Aasebø, E., et al., *Proteomic Characterization of Spontaneous Stress-Induced In Vitro Apoptosis of Human Acute Myeloid Leukemia Cells; Focus on Patient Heterogeneity and Endoplasmic Reticulum Stress.* Hemato, 2021. **2**(3): p. 607–627.
71. Scholl, C., D.G. Gilliland, and S. Frohling, *Deregulation of signaling pathways in acute myeloid leukemia.* Semin Oncol, 2008. **35**(4): p. 336-45.
72. Park, S., et al., *Role of the PI3K/AKT and mTOR signaling pathways in acute myeloid leukemia.* Haematologica, 2010. **95**(5): p. 819-28.
73. Ruvolo, P.P., *GSK-3 as a novel prognostic indicator in leukemia.* Adv Biol Regul, 2017. **65**: p. 26-35.
74. Ruvolo, P.P., et al., *Phosphorylation of GSK3alpha/beta correlates with activation of AKT and is prognostic for poor overall survival in acute myeloid leukemia patients.* BBA Clin, 2015. **4**: p. 59-68.
75. Braess, J., et al., *Cytidine deaminase - the methodological relevance of AraC deamination for ex vivo experiments using cultured cell lines, fresh leukemic blasts, and normal bone marrow cells.* Ann Hematol, 1999. **78**(11): p. 514-20.
76. Gao, G. and Q.P. Dou, *N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death.* J Cell Biochem, 2000. **80**(1): p. 53-72.
77. Maurer, U., et al., *Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1.* Mol Cell, 2006. **21**(6): p. 749-60.
78. Warren, C.F.A., M.W. Wong-Brown, and N.A. Bowden, *BCL-2 family isoforms in apoptosis and cancer.* Cell Death Dis, 2019. **10**(3): p. 177.
79. Lin, K.H., et al., *Targeting MCL-1/BCL-XL Forestalls the Acquisition of Resistance to ABT-199 in Acute Myeloid Leukemia.* Sci Rep, 2016. **6**: p. 27696.
80. Hird, A.W. and A.E. Tron, *Recent advances in the development of Mcl-1 inhibitors for cancer therapy.* Pharmacol Ther, 2019. **198**: p. 59-67.

81. Cerella, C., M. Dicato, and M. Diederich, *BH3 Mimetics in AML Therapy: Death and Beyond?* Trends Pharmacol Sci, 2020. **41**(11): p. 793-814.
82. Ladikou, E.E., et al., *Acute Myeloid Leukaemia in Its Niche: the Bone Marrow Microenvironment in Acute Myeloid Leukaemia.* Curr Oncol Rep, 2020. **22**(3): p. 27.
83. Meads, M.B., R.A. Gatenby, and W.S. Dalton, *Environment-mediated drug resistance: a major contributor to minimal residual disease.* Nat Rev Cancer, 2009. **9**(9): p. 665-74.
84. Bradstock, K.F. and D.J. Gottlieb, *Interaction of acute leukemia cells with the bone marrow microenvironment: implications for control of minimal residual disease.* Leuk Lymphoma, 1995. **18**(1-2): p. 1-16.
85. Ciciarello, M., et al., *The Yin and Yang of the Bone Marrow Microenvironment: Pros and Cons of Mesenchymal Stromal Cells in Acute Myeloid Leukemia.* Front Oncol, 2019. **9**: p. 1135.
86. Yakymiv, Y., et al., *Expression, Functions and Potential Therapeutic Implications of CD157 in Acute Myeloid Leukemia.* J Stem Cell Res Dev Ther, 2020. **6**(038).
87. Chen, P., et al., *Induction of Multidrug Resistance of Acute Myeloid Leukemia Cells by Cocultured Stromal Cells via Upregulation of the PI3K/Akt Signaling Pathway.* Oncol Res, 2016. **24**(4): p. 215-23.
88. Wang, X., et al., *Chemotherapy-induced differential cell cycle arrest in B-cell lymphomas affects their sensitivity to Wee1 inhibition.* Haematologica, 2018. **103**(3): p. 466-476.
89. Hernandez-Campo, P.M., et al., *Quantitative analysis of the expression of glycosylphosphatidylinositol-anchored proteins during the maturation of different hematopoietic cell compartments of normal bone marrow.* Cytometry B Clin Cytom, 2007. **72**(1): p. 34-42.
90. Liang, F., R.Z. Qi, and C.F. Chang, *Signalling of GPI-anchored CD157 via focal adhesion kinase in MCA102 fibroblasts.* FEBS Lett, 2001. **506**(3): p. 207-10.
91. Ngoi, N.Y.L., et al., *Targeting Mitochondrial Apoptosis to Overcome Treatment Resistance in Cancer.* Cancers (Basel), 2020. **12**(3).
92. Ricciardi, M.R., et al., *Targeting the Akt, GSK-3, Bcl-2 axis in acute myeloid leukemia.* Adv Biol Regul, 2017. **65**: p. 36-58.
93. Hers, I., E.E. Vincent, and J.M. Tavare, *Akt signalling in health and disease.* Cell Signal, 2011. **23**(10): p. 1515-27.
94. Toker, A. and S. Marmiroli, *Signaling specificity in the Akt pathway in biology and disease.* Adv Biol Regul, 2014. **55**: p. 28-38.
95. Dai, H., et al., *Constitutive BAK activation as a determinant of drug sensitivity in malignant lymphohematopoietic cells.* Genes Dev, 2015. **29**(20): p. 2140-52.
96. Yakymiv, Y., et al., *CD157 signaling promotes survival of acute myeloid leukemia cells and modulates sensitivity to cytarabine through regulation of anti-apoptotic Mcl-1.* Sci Rep, 2021. **11**(1): p. 21230.
97. Ramsey, H.E., et al., *A Novel MCL1 Inhibitor Combined with Venetoclax Rescues Venetoclax-Resistant Acute Myelogenous Leukemia.* Cancer Discov, 2018. **8**(12): p. 1566-1581.
98. Pei, S., et al., *Monocytic Subclones Confer Resistance to Venetoclax-Based Therapy in Patients with Acute Myeloid Leukemia.* Cancer Discov, 2020. **10**(4): p. 536-551.
99. Macanas-Pirard, P., et al., *Resistance of leukemia cells to cytarabine chemotherapy is mediated by bone marrow stroma, involves cell-surface*

equilibrative nucleoside transporter-1 removal and correlates with patient outcome. Oncotarget, 2017. **8**(14): p. 23073-23086.
100. Bendall, L.J., et al., *Stem cell factor enhances the adhesion of AML cells to fibronectin and augments fibronectin-mediated anti-apoptotic and proliferative signals.* Leukemia, 1998. **12**(9): p. 1375-82.
101. Garrido, S.M., et al., *Acute myeloid leukemia cells are protected from spontaneous and drug-induced apoptosis by direct contact with a human bone marrow stromal cell line (HS-5).* Exp Hematol, 2001. **29**(4): p. 448-57.
102. Selby, C., L.R. Yacko, and A.E. Glode, *Gemtuzumab Ozogamicin: Back Again.* J Adv Pract Oncol, 2019. **10**(1): p. 68-82.